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80538 München (DE)(54) **VARIANT PHOSPHOENOLPYRUVATE CARBOXYLASE, GENE THEREOF, AND PROCESS FOR PRODUCING AMINO ACID**

(57) A variant phosphoenolpyruvate carboxylase that is not substantially inhibited by aspartic acid is produced by introducing a variant phosphoenolpyruvate carboxylase gene, such as one wherein the 625th glutamic acid residue from the N-terminus of the carboxylase has been replaced by a lysine residue or one wherein the 438th arginine residue has been replaced by a cysteine residue, into *Escherichia coli* or a coryneform bacterium. An amino acid can efficiently be produced by using this carboxylase.

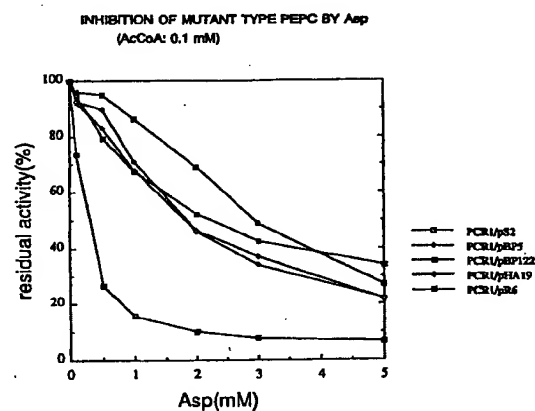


Fig. 9

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## Description

TECHNICAL FIELD

5 The present invention relates to a mutant phosphoenolpyruvate carboxylase, a gene coding for it, and a production method of an amino acid, and in particular relates to a gene having mutation to desensitize feedback inhibition by aspartic acid, and utilization thereof.

BACKGROUND ART

10 Phosphoenolpyruvate carboxylase is an enzyme which is found in almost all bacteria and all plants. The role of this enzyme resides in biosynthesis of aspartic acid and glutamic acid, and supply of C4 dicarboxylic acid to the citric acid cycle for maintaining its turnover. However, in the fermentative production of an amino acid using a microorganisms, there have been few reports on effects of this enzyme has not been made clear (Atsushi Yokota and Isamu Shiio, Agric. Biol. Chem., 52, 455-463 (1988), Josef Cremer et al., Appl. Environ. Microbiol., 57, 1746-1752 (1991), Petra, G. Peters-Weintisch, FEMS Microbiol. Letters, 112, 269-274 (1993)).

15 By the way, the amino acid is a compound which universally exists in cells as components of proteins, however, for the sake of economic energy metabolism and substance metabolism, its production is strictly controlled. This control is principally feedback control, in which the final product of a metabolic pathway inhibits the activity of an enzyme which catalyzes the earlier step of the pathway. Phosphoenolpyruvate carboxylase also undergoes various regulations in expression of its activity.

20 For example, in the case of phosphoenolpyruvate carboxylase of microorganisms belonging to the genus Corynebacterium or the genus Escherichia, the activity is inhibited by aspartic acid. Therefore, the aforementioned amino acid biosynthesis, in which phosphoenolpyruvate carboxylase participates, is also inhibited by aspartic acid.

25 In the prior art, various techniques have been developed for efficient production in amino acid fermentation, and fermentative production has been carried out for leucine, isoleucine, tryptophan, phenylalanine and the like by using mutant strains converted to be insensitive to feedback control. However, there has been known neither mutant phosphoenolpyruvate carboxylase converted to be insensitive to inhibition by aspartic acid, nor attempt to utilize it for fermentative production of amino acids of the aspartic acid family and the glutamic acid family.

30 On the other hand, ppc gene, which is a gene coding for phosphoenolpyruvate carboxylase of Escherichia coli, has been already cloned, and also determined for its nucleotide sequence (Fujita, N., Miwa, T., Ishijima, S., Izui, K. and Katsuki, H., J. Biochem., 95, 909-916 (1984)). However, there is no report of a mutant in which inhibition by aspartic acid is desensitized.

35 The present invention has been made from the aforementioned viewpoint, an object of which is to provide a mutant phosphoenolpyruvate carboxylase with substantially desensitized feedback inhibition by aspartic acid, a gene coding for it, and a utilization method thereof.

DISCLOSURE OF THE INVENTION

40 As a result of diligent investigation in order to achieve the aforementioned object, the present inventors have found that the inhibition by aspartic acid is substantially desensitized by replacing an amino acid at a specified site of phosphoenolpyruvate carboxylase of Escherichia coli with another amino acid, succeeded in obtaining a gene coding for such a mutant enzyme, and arrived at completion of the present invention.

45 Namely, the present invention lies in a mutant phosphoenolpyruvate carboxylase, which originates from a microorganism belonging to the genus Escherichia, and has mutation to desensitize feedback inhibition by aspartic acid, and a DNA sequence coding for the mutant phosphoenolpyruvate carboxylase.

50 The present invention further provides microorganisms belonging to the genus Escherichia or coryneform bacteria harboring the DNA fragment, and a method of producing an amino acid wherein any of these microorganisms is cultivated in a preferable medium, and the amino acid selected from L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamic acid, L-arginine and L-proline is separated from the medium.

Incidentally, in this specification, the DNA sequence coding for the mutant phosphoenolpyruvate carboxylase, or a DNA sequence containing a promoter in addition thereto is occasionally merely referred to as "DNA sequence of the present invention", "mutant gene" or "phosphoenolpyruvate carboxylase gene."

The present invention will be explained in detail hereinafter.

## (1) Mutant phosphoenolpyruvate carboxylase

The mutant phosphoenolpyruvate carboxylase of the present invention (hereinafter simply referred to as "mutant enzyme") lies in the phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia, which has mutation to desensitize the feedback inhibition by aspartic acid.

Such mutation may be any one provided that the aforementioned feedback inhibition is substantially desensitized without losing the enzyme activity of the phosphoenolpyruvate carboxylase, for which there may be exemplified mutation which, when a mutant phosphoenolpyruvate carboxylase having the mutation is allowed to exist in cells of a microorganism belonging to the genus Escherichia, gives the cells resistance to a compound having the following properties:

it exhibits a growth inhibitory action against a microorganism belonging to the genus Escherichia which produces a wild type phosphoenolpyruvate carboxylase;

the aforementioned growth inhibitory action is recovered by existence of L-glutamic acid or L-aspartic acid; and it inhibits wild type phosphoenolpyruvic carboxylase activity.

More concretely, there may be exemplified, as counted from the N-terminus of the phosphoenolpyruvate carboxylase:

- (1) mutation to replace 625th glutamic acid with lysine;
- (2) mutation to replace 222th arginine with histidine and 223th glutamic acid with lysine, respectively;
- (3) mutation to replace 288th serine with phenylalanine, 289th glutamic acid with lysine, 551th methionine with isoleucine and 804th glutamic acid with lysine, respectively;
- (4) mutation to replace 867th alanine with threonine;
- (5) mutation to replace 438th arginine with cysteine; and
- (6) mutation to replace 620th lysine with serine.

Incidentally, as the phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia, an amino acid sequence, which is deduced from a phosphoenolpyruvate carboxylase gene of Escherichia coli (Fujita, N., Miwa, T., Ishijima, S., Izui, K. and Katsuki, H., J. Biochem., 95, 909-916 (1984)), is shown in SEQ ID NO:2 in the Sequence listing. In addition, an entire nucleotide sequence of a plasmid pT2, which contains the phosphoenolpyruvate carboxylase gene of Escherichia coli, is shown in SEQ ID NO:1 together with the amino acid sequence.

The aforementioned mutant enzymes are encoded by DNA sequences of the present invention described below, which are produced by expressing the DNA sequences in Escherichia coli and the like.

## (2) DNA sequence of the present invention and microorganisms harboring the same

The DNA sequence of the present invention is DNA sequences coding for the aforementioned mutant enzymes, and has mutation to desensitize feedback inhibition of phosphoenolpyruvate carboxylase by aspartic acid in coding regions in DNA fragments coding for phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia.

Concretely, there may be exemplified a DNA Sequence coding for the phosphoenolpyruvate carboxylase having the mutation of any one of the aforementioned (1) to (6), for example, with respect to the nucleotide sequence of SEQ ID NO:1, there may be exemplified a DNA sequence having any one of:

- i) mutation to convert GAA of base Nos. 2109-2111 into AAA or AAG;
- ii) mutation to convert CGC of base Nos. 900-902 into CAT or CAC, and GAA of 903-905 into AAA or AAG, respectively;
- iii) mutation to convert TCT of base Nos. 1098-1100 into TTT or TTC, GAA of 1101-1103 into AAA or AAG, ATG of 1887-1889 into ATT, ATC or ATA, and GAA of 2646-2648 into AAA or AAG, respectively;
- iv) mutation to convert GCG of 2835-2837 into any one of ACT, ACC, ACA and ACG; and
- v) mutation to convert CGT of 1548-1550 into TGT or TGC; and
- vi) mutation to convert AAA of 2094-2096 into TCT, TCC, TCA or TCG.

Such a mutant gene is obtained such that a recombinant DNA, which is obtained by ligating a phosphoenolpyruvate carboxylase gene as a wild type enzyme gene or having another mutation with a vector DNA adaptable to a host, is subjected to a mutation treatment, to perform screening from transformants by the recombinant DNA. Alternatively, it is also acceptable that a microorganism which produces a wild type enzyme is subjected to a mutation treatment, a mutant strain which produces a mutant enzyme is created, and then a mutant gene is screened from the mutant strain. For the mutation treatment of the recombinant DNA, hydroxylamine and the like may be used. Further, when an microorganism itself is subjected to a mutation treatment, a drug or a method usually used for artificial mutation may be used.

Further, in accordance with methods such as the Overlapping Extension method (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., Gene, 77, 51-59 (1989)), the site specific mutation method (Kramer, W. and Frits, H. J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T. A. et al., Meth. in Enzymol., 154, 367 (1987)) and the like, the aforementioned mutant gene can be also obtained by introducing mutation such as amino acid replacement, insertion, deletion and the like into a phosphoenolpyruvate carboxylase gene as a wild type enzyme gene or having another mutation. These methods are based on a principle that a non-mutated gene DNA is used as a template, and a synthetic DNA containing a mismatch at a mutation point is used as one of primers so as to synthesize complementary strands of the aforementioned gene DNA, thereby mutation is introduced. By using these methods, it is possible to cause intended mutation at an aimed site.

Alternatively, a sequence, which has restriction enzyme cleavage ends at both termini and includes both sides of a mutation point, is synthesized, and exchanged for a corresponding portion of a non-mutated gene, thereby mutation can be introduced (cassette mutation method).

The phosphoenolpyruvate carboxylase gene, which is the wild type enzyme gene or has another mutation to be used for introduction of mutation, may be any one provided that it is a gene coding for the phosphoenolpyruvate carboxylase of the microorganism belonging to the genus Escherichia, which is preferably determined for its base sequence and cloned. When it has not been cloned, a DNA fragment containing the gene can be amplified and isolated by using the PCR method and the like, followed by using a suitable vector to achieve cloning.

As the gene as described above, for example, there may be exemplified a gene of Escherichia coli having been cloned and determined for its base sequence (Fujita, N., Miwa, T., Ishijima, S., Izui, K. and Katsuki, H., J. Biochem., 95, 909-916 (1984)). The sequence in the coding region of this gene is as shown in SEQ ID NO: 1 (base Nos. 237-2888).

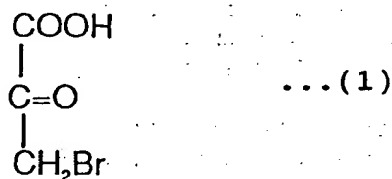
Screening of a host harboring the mutant gene can be performed by using an analog compound of aspartic acid. The analog compound preferably has the following properties. Namely, it exhibits a growth inhibitory action against a microorganism belonging to the genus Escherichia which produces a wild type phosphoenolpyruvate carboxylase, the aforementioned growth inhibitory action is recovered by existence of L-glutamic acid or L-aspartic acid, and it inhibits wild type phosphoenolpyruvate carboxylase activity.

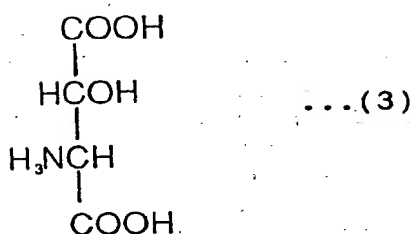
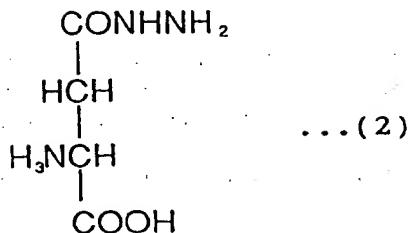
If a mutant strain being resistant to the analog compound mentioned above is selected from microorganism belonging to the genus Escherichia, for example, Escherichia coli HB101 producing wild type phosphoenolpyruvate carboxylase using inhibition of growth of the microorganism as an index, it is much likely to obtain a host microorganism which produces phosphoenolpyruvate carboxylase with desensitized feedback inhibition by aspartic acid.

It is proposed, as a general structure of an inhibitor of phosphoenolpyruvate carboxylase, that a C4 dicarboxylic acid structure is essentially provided. From such a viewpoint, various compounds were subjected to screening by the present inventors. Escherichia coli HB101 was cultivated in an LB medium, and transferred to M9 media (containing 20 µg/ml of thiamine and 3 µg/ml of each of Leu and Pro) containing any one of DL-2-amino-4-phosphonobutyric acid, bromosuccinic acid, meso-2,3-dibromosuccinic acid, 2,2-difluorosuccinic acid, 3-bromopyruvic acid, α-ketobutyric acid, α-ketoadipinic acid, DL-threo-β-hydroxyaspartic acid, L-aspartic acid β-methyl ester, α-methyl-DL-aspartic acid, 2,3-diaminosuccinic acid or aspartic acid-β-hydrazide, and absorbance of the medium was measured at 660 nm with the passage of time, thereby growth was monitored.

Further, when these compounds were present at their growth inhibitory concentrations, it was investigated whether or not the inhibition was recovered by addition of nucleic acids (each of uridine, adenosine: 10 mg/dl), glutamic acid or amino acids of the aspartic acid family (Asp: 0.025 %, each of Met, Thr, Lys: 0.1 %).

As a result, three compounds: 3-bromopyruvate (3BP) (1), aspartate-β-hydrazide (AHY) (2), and DL-threo-β-hydroxyaspartate (βHA) (3) were selected.





Growth inhibition of *Escherichia coli* by these analog compounds is shown in Figs. 1-3. Further, growth recovery of *Escherichia coli*, in the case of addition of the aforementioned inhibition recovering substances alone or as a mixture of 2 species or 3 species, is shown in Figs. 4-6. In addition, as a control, growth in the case of addition of the inhibition recovering substance in the absence of the inhibitory substance is shown in Fig. 7. Incidentally, in Figs. 4-7, additives 1, 2 and 3 indicate nucleic acids, glutamic acid or amino acids of the aspartic acid family, respectively.

Further, inhibition of activity by the analog compound on phosphoenolpyruvate carboxylase was investigated. Crude enzyme was prepared from an *Escherichia coli* HB101 strain in accordance with a method described in *The Journal of Biochemistry*, Vol. 67, No. 4 (1970), and enzyme activity was measured in accordance with a method described in *Eur. J. Biochem.*, 202, 797-803 (1991).

*Escherichia coli* HB101 cultivated in an LB medium was disrupted, and a suspension was centrifuged to obtain a supernatant which was used as a crude enzyme solution. Measurement of enzyme activity was performed by measuring decrease in absorbance at 340 nm while allowing acetyl-coenzyme A known to affect the activity to exist at a concentration of 0.1 mM in a measurement system containing 2 mM potassium phosphoenolpyruvate, 0.1 mM NADH, 0.1 M Tris-acetate (pH 8.5), 1.5 U malate dehydrogenase and crude enzyme. Results are shown in Fig. 8.

According to the results as above, it is apparent that the aforementioned three compounds inhibit growth of *Escherichia coli*, this inhibition cannot be recovered by nucleic acids alone, but the inhibition can be recovered by addition of glutamic acid or amino acids of the aspartic acid family. Therefore, these analog compounds were postulated to be selective inhibitors of phosphoenolpyruvate carboxylase. As shown in Examples described below, by using these compounds, the present invention has succeeded in selection of an *Escherichia coli* which produces the mutant phosphoenolpyruvate carboxylase.

When a transformant having an aimed mutant enzyme gene is screened by using the aforementioned compounds, and a recombinant DNA is recovered, then the mutant enzyme gene is obtained. Alternatively, in the case of a mutation treatment of an microorganism itself, when a mutant strain having an aimed mutant enzyme gene is screened by using the aforementioned compounds, a DNA fragment containing the aimed mutant enzyme gene is isolated from the strain, and it is ligated with a suitable vector, then the mutant enzyme gene is obtained.

On the other hand, as a result of diligent investigation by the present inventors taking notice of importance of an arginine residue in an aspartate binding protein of *Escherichia coli* (Krikos, A., Mouth, N., Boyd, A. and Simon, M. I. *Cell*, 33, 615-622 (1983), Mowbray, S. L and Koshland, D. E. *J. Biol. Chem.*, 264, 15638-15643 (1990), Milburn, M. V., Prive, G. G., Milligan, D. L., Scott, W. G., Yeh, J., Jancarik, J., Koshland, D. E. and Kim, S. H., *Science*, 254, 1342-1347 (1991)), it has been found that inhibition by aspartic acid is substantially desensitized by converting 438th arginine of phosphoenolpyruvate carboxylase into cysteine. In order to convert 438th arginine into cysteine, a codon of 438th

arginine of a gene coding for phosphoenolpyruvate carboxylase may be converted into a codon of cysteine. For example, in SEQ ID NO:1, CGT of nucleotide numbers of 1548-1550 may be converted into TGT or TGC.

Further, the present inventors performed chemical modification of lysine residues of phosphoenolpyruvate carboxylase by using 2,4,6-trinitrobenzenesulfonic acid (TNBS) which is a compound to chemically modify lysine residues of a protein. During modification reaction, malic acid capable of serving as an inhibitor of phosphoenolpyruvate carboxylase was allowed to exist together. Namely, it was assumed that a lysine residue in the vicinity of a binding position of phosphoenolpyruvate carboxylase would be protected by bound malic acid and not be subjected to chemical modification. As a result, it was suggested that a 620th lysine residue was important for malic acid to bind phosphoenolpyruvate carboxylase, and it was found that the feedback inhibition by aspartic acid was desensitized while maintaining the enzyme activity of phosphoenolpyruvate carboxylase by converting the 620th lysine residue into a serine residue. In order to convert the 620th lysine residue into the serine residue, a codon of 620th lysine of the gene coding for phosphoenolpyruvate carboxylase may be converted into a codon of serine. For example, in SEQ ID NO:1, AAA having nucleotide numbers of 2094-2096 may be replaced with TCT, TCC, TCA, TCG, AGT or AGC.

In accordance with methods such as the Overlapping Extension method (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., Gene, 77, 51-59 (1989)), the site specific mutation method (Kramer, W. and Frits, H. J., Meth. in Enzymol., 154, 350 (1987); Kunkel, T. A. et al., Meth. in Enzymol., 154, 367 (1987)) and the like, conversion of the codon can be also achieved by introducing mutation such as amino acid replacement, insertion, deletion and the like into a phosphoenolpyruvate carboxylase gene as a wild type enzyme gene or having another mutation. These methods are based on a principle that a non-mutated gene DNA is used as a template, and a synthetic DNA containing a mismatch at a mutation point is used as one of primers so as to synthesize complementary strands of the aforementioned gene DNA, thereby mutation is introduced. By using these methods, it is possible to cause intended mutation at an aimed site.

Alternatively, a sequence, which has restriction enzyme cleavage ends at both termini and contains both sides of a mutation point, is synthesized, and exchanged for a corresponding portion of a non-mutated gene, thereby mutation can be introduced (cassette mutation method).

The DNA fragment coding for the phosphoenolpyruvate carboxylase with mutation introduced as described above is expressed by using a suitable host-vector system, thereby it is possible to produce a mutant enzyme. Alternatively, even by performing transformation by integrating the DNA fragment of the present invention into a host chromosomal DNA, an aimed mutant enzyme can be produced.

As the host, there may be exemplified microorganisms belonging to the genus Escherichia, for example, Escherichia coli, coryneform bacteria and the like. The coryneform bacteria include bacteria belonging to the genus Corynebacterium, bacteria belonging to the genus Brevibacterium having been hitherto classified into the genus Brevibacterium but being united as bacteria belonging to the genus Corynebacterium at present, and bacteria belonging to the genus Brevibacterium closely related to bacteria belonging to the genus Corynebacterium. Incidentally, hosts which are preferable for amino acid production will be described below.

On the other hand, as the vector DNA, a plasmid vector is preferable, and those capable of self-replication in a host cell are preferable. When the host is Escherichia coli, for example, pUC19, pUC18, pBR322, pHSG299, pHSG399, RSF1010 and the like are exemplified. Alternatively, a vector of phage DNA can be also utilized.

Further, when the host is the coryneform bacteria, vectors which can be used and hosts which harbor them are exemplified below. Incidentally, deposition numbers of international depositories are shown in parentheses.

pAJ655 Escherichia coli AJ11882 (FERM BP-136)  
Corynebacterium glutamicum SR8201 (ATCC 39135)  
pAJ1844 Escherichia coli AJ11883 (FERM BP-137)  
Corynebacterium glutamicum SR8202 (ATCC 39136)  
pAJ611 Escherichia coli AJ11884 (FERM BP-138)  
pAJ3148 Corynebacterium glutamicum SR8203 (ATCC 39137)  
pAJ440 Bacillus subtilis AJ11901 (FERM BP-140)

These vectors may be obtained from the deposited microorganisms as follows. Cells collected at the logarithmic growth phase are subjected to bacteriolysis by using lysozyme and SDS, and centrifuged at 30000 x g to obtain a supernatant solution from a lysate, to which polyethylene glycol is added to perform separation and purification of the vectors by means of cesium chloride-ethidium bromide equilibrium density gradient centrifugation.

In order to transform Escherichia coli with a recombinant vector obtained by inserting the DNA sequence of the present invention into the aforementioned vector, it is possible to use a method usually used for transformation of Escherichia coli, such as a method in which cells are treated with calcium chloride to enhance permeability of DNA (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1977)) and the like.

Further, as a method for transforming the coryneform bacteria, there is the aforementioned method in which cells are treated with calcium chloride, or a method in which incorporation is performed at a specified growth period in which

cells can incorporate DNA (report in relation to Bacillus subtilis by Duncan, C. H. et al.). Further, incorporation into bacterial cells can be achieved by forming protoplasts or spheroplasts of DNA recipients which easily incorporate plasmid DNA. These are known for Bacillus subtilis, Actinomyces and yeast (Chang, S. et al., Molec. Gen. Genet., 168, 111 (1979), Bibb et al., Nature, 274, 398 (1978), Hinnen, A. et al., Proc. Natl. Acad. Sci. USA, 75 1929 (1978)). Additionally, a method for transforming coryneform bacteria is disclosed in Japanese Patent Laid-open No. 2-207791.

In order to express the DNA sequence of the present invention in the aforementioned hosts, a promoter such as lac, trp, PL and the like which efficiently works in microorganisms may be used, or when the DNA sequence of the present invention contains a promoter of the phosphoenolpyruvate carboxylase gene, it may be used as it is. Alternatively, when the coryneform bacterium is used as the host, it is also possible to use a known trp promoter originating from a bacterium belonging to the genus Brevibacterium (Japanese Patent Laid-open No. 62-244382) and the like.

Further, as described above, it is acceptable that the DNA sequence of the present invention is inserted into the vector DNA capable of self-replication and introduced into the host to allow the host to harbor it as a plasmid, and it is also acceptable that the DNA sequence of the present invention is integrated into a chromosome of a microorganism by means of a method using transposon (Berg, D. E. and Berg, C. M., Bio/Technol., 1, 417 (1983)), Mu phage (Japanese Patent Laid-open No. 2-109985) or homologous recombination (Experiments in Molecular Genetics, Cold Spring Harbor Lab. (1972)). In addition, in order to integrate the DNA of the present invention into the coryneform bacteria, it is possible to utilize a temperature-sensitive plasmid disclosed in Japanese Patent Laid-open No. 5-7491.

When the microorganism transformed with the DNA sequence of the present invention as described above is cultivated, and this DNA sequence is expressed, then a mutant enzyme is obtained. It becomes apparent, by measuring the activity by adding aspartic acid to an enzyme reaction system, whether or not the mutant enzyme thus obtained has desensitized feedback inhibition by aspartic acid. It is possible for the measurement of the enzyme activity to use a spectrometric method (Yoshinaga, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (1970)) and the like.

Further, the DNA sequence of the present invention codes for the mutant enzyme in which feedback inhibition by aspartic acid is desensitized, so that the microorganism harboring this DNA sequence can be utilized for efficient fermentative production of amino acids of the aspartic acid family and the glutamic acid family as described below.

Escherichia coli AJ12907, AJ12908, AJ12909 and AJ12910 harboring the mutant enzyme genes obtained in Examples described below are deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on August 3, 1993 under the deposition numbers of FERM P-13774, FERM P-13775, FERM P-13776 and FERM P-13777, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition numbers of FERM BP-4734, FERM BP-4735, FERM BP-4736, FERM BP-4737, respectively in this order.

### (3) Production method of amino acids

Amino acids can be produced by cultivating the microorganism harboring the DNA sequence of the present invention in a preferable medium, and separating generated amino acids. As such amino acids, there may be exemplified L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamic acid, L-arginine and L-proline.

Preferable hosts into which the DNA sequence of the present invention is introduced to be used for production of each of the amino acids, and a cultivation method will be exemplified below.

#### (1) Hosts preferable for the amino acid production method of the present invention

##### (i) Hosts preferable for L-lysine production

As the host to be used for L-lysine production according to the present invention, there may be exemplified bacteria belonging to the genus Escherichia, preferably L-lysine-producing Escherichia coli. Concretely, a mutant strain having resistance to a lysine analog can be exemplified. Such a lysine analog is those which inhibit growth of microorganisms belonging to the genus Escherichia, however, the suppression is totally or partially desensitized provided that L-lysine co-exists in the medium. For example, there are oxalysine, lysine hydroxamate, S-(2-aminoethyl)-cysteine (hereinafter abbreviated as "AEC"),  $\gamma$ -methyllysine,  $\alpha$ -chlorocaprolactam and the like. Mutant strains having resistance to these lysine analogs can be obtained by applying an ordinary artificial mutation treatment to microorganisms belonging to the genus Escherichia. Concretely, as a bacterial strain to be used for L-lysine production, there may be exemplified Escherichia coli AJ11442 (deposited as FERM P-5084, see lower-left column on page 471 in Japanese Patent Laid-open No. 56-18596).

On the other hand, various artificial mutant strains of coryneform bacteria which have been used as L-lysine-producing bacteria can be used for the present invention. Such artificial mutant strains are as follows: AEC resistant mutant strain; mutant strain which requires amino acid such as L-homoserine for its growth (Japanese Patent Publication Nos. 48-28078 and 56-6499); mutant strain which exhibits resistance to AEC and requires amino acid such as L-leucine, L-

homoserine, L-proline, L-serine, L-arginine, L-alanine, L-valine and the like (United States Patent Nos. 3708395 and 3825472); L-lysine-producing mutant strain which exhibits resistance to DL- $\alpha$ -amino- $\epsilon$ -caprolactam,  $\alpha$ -amino-lauryl-lactam, quinoid and N-lauroylleucine; L-lysine-producing mutant strain which exhibits resistance to an inhibitor of oxaloacetate decarboxylase or respiratory system enzyme (Japanese Patent Laid-open Nos. 50-53588, 50-31093, 52-102498, 53-86089, 55-9783, 55-9759, 56-32995 and 56-39778, and Japanese Patent Publication Nos. 53-43591 and 53-1833); L-lysine-producing mutant strain which requires inositol or acetic acid (Japanese Patent Laid-open Nos. 55-9784 and 56-8692); L-lysine-producing mutant strain which exhibits sensitivity to fluoropyruvate or temperature not less than 34 °C (Japanese Patent Laid-open Nos. 55-9783 and 53-86090); and mutant strain of Brevibacterium or Corynebacterium which exhibits resistance to ethylene glycol and produces L-lysine (see United States Patent Application Serial No. 333455).

Followings are exemplified as concrete coryneform bacteria to be used for lysine production:

Brevibacterium lactofermentum AJ12031 (FERM-BP277), see page 525 in Japanese Patent Laid-open No. 60-62994;

Brevibacterium lactofermentum ATCC 39134, see lower-right column on page 473 in Japanese Patent Laid-open No. 60-62994;

Brevibacterium lactofermentum AJ3463 (FERM-P1987), see Japanese Patent Publication No. 51-34477.

In addition, wild strains of coryneform bacteria described below can be also used for the present invention in the same manner.

Corynebacterium acetoacidophilum  
ATCC 13870

Corynebacterium acetoglutamicum  
ATCC 15806

Corynebacterium callunae  
ATCC 15991

Corynebacterium glutamicum  
ATCC 13032  
ATCC 13060

(Brevibacterium divaricatum)

ATCC 14020

(Brevibacterium lactofermentum)

ATCC 13869

(Corynebacterium lilium)

ATCC 15990

Corynebacterium melassecola

ATCC 17965

Brevibacterium saccharolyticum

ATCC 14066

Brevibacterium immariophilum

ATCC 14068

Brevibacterium roseum

ATCC 13825

Brevibacterium flavum

ATCC 13826

Brevibacterium thioenitalis

ATCC 19240

Microbacterium ammoniophilum

ATCC 15354

(ii) Hosts preferable for L-threonine production

Escherichia coli B-3996 (RIA 1867), see Japanese Patent Laid-open No. 3-501682 (PCT);

Escherichia coli AJ12349 (FERM-P9574), see upper-left column on page 887 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12351 (FERM-P9576), see lower-right column on page 887 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ12352 (FERM P-9577), see upper-left column on page 888 in Japanese Patent Laid-open No. 2-458;

Escherichia coli AJ11332 (FERM P-4898), see upper-left column on page 889 in Japanese Patent Laid-open No.



2-458;  
Escherichia coli AJ12350 (FERM P-9575), see upper-left column on page 889 in Japanese Patent Laid-open No.  
 2-458;  
Escherichia coli AJ12353 (FERM P-9578), see upper-right column on page 889 in Japanese Patent Laid-open  
 5 No. 2-458;  
Escherichia coli AJ12358 (FERM P-9764), see upper-left column on page 890 in Japanese Patent Laid-open No.  
 2-458;  
Escherichia coli AJ12359 (FERM P-9765), see upper-left column on page 890 in Japanese Patent Laid-open No.  
 2-458;  
 10 Escherichia coli AJ11334 (FERM P-4900), see column 6 on page 201 in Japanese Patent Publication No. 1-  
 29559;  
Escherichia coli AJ11333 (FERM P-4899), see column 6 on page 201 in Japanese Patent Publication No. 1-  
 29559;  
Escherichia coli AJ11335 (FERM P-4901), see column 7 on page 202 in Japanese Patent Publication No. 1-  
 15 29559.

Following bacterial strains are exemplified as the coryneform bacteria:

Brevibacterium lactofermentum AJ11188 (FERM P-4190), see upper-right column on page 473 in Japanese Pat-  
 ent Laid-open No. 60-87788;  
Corynebacterium glutamicum AJ11682 (FERM BP-118), see column 8 on page 230 in Japanese Patent Publi-  
 20 cation No. 2-31956;  
Brevibacterium flavum AJ11683 (FERM BP-119), see column 10 on page 231 in Japanese Patent Publication  
 No. 2-31956.

(iii) Hosts preferable for L-methionine production

Following bacterial strains are exemplified for L-methionine production:

Escherichia coli AJ11457 (FERM P-5175), see upper-right column on page 552 in Japanese Patent Laid-open  
 No. 56-35992;  
Escherichia coli AJ11458 (FERM P-5176), see upper-right column on page 552 in Japanese Patent Laid-open  
 30 No. 56-35992;  
Escherichia coli AJ11459 (FERM P-5177), see upper-right column on page 552 in Japanese Patent Laid-open  
 No. 56-35992;  
Escherichia coli AJ11539 (FERM P-5479), see lower-left column on page 435 in Japanese Patent Laid-open No.  
 56-144092;  
 35 Escherichia coli AJ11540 (FERM P-5480), see lower-left column on page 435 in Japanese Patent Laid-open No.  
 56-144092;  
Escherichia coli AJ11541 (FERM P-5481), see lower-left column on page 435 in Japanese Patent Laid-open No.  
 56-144092;  
Escherichia coli AJ11542 (FERM P-5482), see lower-left column on page 435 in Japanese Patent Laid-open No.  
 40 56-144092.

(iv) Hosts preferable for L-aspartic acid production

Following bacterial strains are exemplified for L-aspartic acid production:

Brevibacterium flavum AJ3859 (FERM P-2799), see upper-left column on page 524 in Japanese Patent Laid-  
 45 open No. 51-61689;  
Brevibacterium lactofermentum AJ3860 (FERM P-2800), see upper-left column on page 524 in Japanese Patent  
 Laid-open No. 51-61689;  
Corynebacterium acetoacidophilum AJ3877 (FERM-P2803), see upper-left column on page 524 in Japanese  
 50 Patent Laid-open No. 51-61689;  
Corynebacterium glutamicum AJ3876 (FERM P-2802), see upper-left column on page 524 in Japanese Patent  
 Laid-open No. 51-61689.

(v) Hosts preferable for L-isoleucine production

Escherichia coli KX141 (VKPM-B4781) (see 45th paragraph in Japanese Patent Laid-open No. 4-33027) is exem-  
 55 plified as the bacteria belonging to the genus Escherichia, and Brevibacterium lactofermentum AJ12404 (FERM P-  
 10141) (see lower-left column on page 603 in Japanese Patent Laid-open No. 2-42988) and Brevibacterium flavum

AJ12405 (FERM P-10142) (see lower-left column on page 524 in Japanese Patent Laid-open No. 2-42988) are exemplified as the coryneform bacteria.

(vi) Hosts preferable for L-glutamic acid production

Following bacterial strains are exemplified as the bacteria belonging to the genus Escherichia:

Escherichia coli AJ12628 (FERM P-12380), see French Patent Publication No. 2 680 178 (1993);

Escherichia coli AJ12624 (FERM P-12379), see French Patent Publication No. 2 680 178 (1993).

Following bacterial strains are exemplified as the coryneform bacteria:

Brevibacterium lactofermentum AJ12745 (FERM BP-2922), see lower-right column on page 561 in Japanese Patent Laid-open No. 3-49690;

Brevibacterium lactofermentum AJ12746 (FERM BP-2923), see upper-left column on page 562 in Japanese Patent Laid-open No. 3-49690;

Brevibacterium lactofermentum AJ12747 (FERM BP-2924), see upper-left column on page 562 in Japanese Patent Laid-open No. 3-49690;

Brevibacterium lactofermentum AJ12748 (FERM BP-2925), see upper-left column on page 562 in Japanese Patent Laid-open No. 3-49690;

Brevibacterium flavum ATCC 14067, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793;

Corynebacterium glutamicum ATCC 21492, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793.

(vii) Hosts preferable for L-arginine production

Following bacterial strains are exemplified as the bacteria belonging to the genus Escherichia:

Escherichia coli AJ11593 (FERM P-5616), see upper-left column on page 468 in Japanese Patent Laid-open No. 57-5693;

Escherichia coli AJ11594 (FERM P-5617), see upper-right column on page 468 in Japanese Patent Laid-open No. 57-5693.

Following bacterial strains are exemplified as the coryneform bacteria:

Brevibacterium flavum AJ12144 (FERM P-7642), see column 4 on page 174 in Japanese Patent Publication No. 5-27388;

Corynebacterium glutamicum AJ12145 (FERM P-7643), see column 4 on page 174 in Japanese Patent Publication No. 5-27388;

Brevibacterium flavum ATCC 21493, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793;

Corynebacterium glutamicum ATCC 21659, see Table 1 on page 3 in Japanese Patent Laid-open No. 5-3793.

(viii) Hosts preferable for L-proline production

Following bacterial strains are exemplified as the bacteria belonging to the genus Escherichia:

Escherichia coli AJ11543 (FERM P-5483), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144093;

Escherichia coli AJ11544 (FERM P-5484), see lower-left column on page 435 in Japanese Patent Laid-open No. 56-144093.

Following bacterial strains are exemplified as the coryneform bacteria:

Brevibacterium lactofermentum AJ11225 (FERM P-4370), see upper-left column on page 473 in Japanese Patent Laid-open No. 60-87788;

Brevibacterium flavum AJ11512 (FERM P-5332), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;

Brevibacterium flavum AJ11513 (FERM P-5333), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;

Brevibacterium flavum AJ11514 (FERM P-5334), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;

Corynebacterium glutamicum AJ11522 (FERM P-5342), see column 2 on page 185 in Japanese Patent Publication No. 62-36679;

Corynebacterium glutamicum AJ11523 (FERM P-5343), see column 2 on page 185 in Japanese Patent Publication No. 62-36679.

## (2) Cultivation method

The method for cultivating the aforementioned hosts is not especially different from a cultivation method for amino acid-producing microorganisms in the prior art. Namely, an ordinary medium is used containing a carbon source, a nitrogen source and inorganic ions, and optionally organic trace nutrients such as amino acids, vitamins and the like.

As the carbon source, glucose, sucrose, lactose and the like, as well as starch hydrolysate, whey, molasses and the like containing them may be used. As the nitrogen source, ammonia gas, aqueous ammonium, ammonium salt and the like can be used. Incidentally, when a nutrient requiring mutant strain for amino acids or the like is used as the host, it is necessary to suitably add the nutrient such as amino acid or the like required by the strain to the medium. An example of the medium for lysine production is shown in Table 1 below as a medium to be used for amino acid production. Incidentally, calcium carbonate is added to other components after being separately sterilized.

Table 1

Medium component	Blending amount
glucose	5 g/dl
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	2.5 g/dl
KH <sub>2</sub> PO <sub>4</sub>	0.2 g/dl
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.1 g/dl
yeast extract	0.05 g/dl
thiamine hydrochloride	1 µg/l
biotin	300 µg/l
FeSO <sub>4</sub> · 7H <sub>2</sub> O	1 mg/dl
MnSO <sub>4</sub> · 4H <sub>2</sub> O	1 mg/dl
calcium carbonate	2.5 g/dl
(pH 7.0)	

The cultivation is performed until generation and accumulation of amino acids substantially stop while suitably controlling pH and temperature of the medium under an aerobic condition. In order to collect amino acids thus accumulated in the cultivated medium, an ordinary method can be applied.

BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1 shows growth inhibition by 3-bromopyruvate.  
 Fig. 2 shows growth inhibition by aspartate-β-hydrazide.  
 Fig. 3 shows growth inhibition by DL-threo-β-hydroxyaspartate.  
 Fig. 4 shows effects of inhibition recovering substances on 3-bromopyruvate.  
 Fig. 5 shows effects of inhibition recovering substances on aspartate-β-hydrazide.  
 Fig. 6 shows effects of inhibition recovering substances on DL-threo-β-hydroxyaspartate.  
 Fig. 7 shows influences exerted on growth by growth recovering factors.  
 Fig. 8 shows inhibition of phosphoenolpyruvate carboxylase by growth inhibitory substances.  
 Fig. 9 shows inhibition of phosphoenolpyruvate carboxylase of the present invention by aspartic acid.  
 Fig. 10 shows inhibition of phosphoenolpyruvate carboxylase of the present invention by aspartic acid.  
 Fig. 11 shows a method for introducing mutation into a phosphoenolpyruvate carboxylase gene.  
 Fig. 12 shows influences exerted by aspartic acid on activities of wild type and mutant phosphoenolpyruvate carboxylase in which 438th arginine was substituted with cysteine counted from the N-terminus.  
 Fig. 13 shows influences exerted by aspartic acid on activities of wild type and mutant phosphoenolpyruvate carboxylase in which 620th lysine was substituted with serine counted from the N-terminus.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will be explained more concretely below with reference to Examples.

Example 1: acquisition of mutant phosphoenolpyruvate carboxylase gene

A mutant gene was prepared by using a plasmid pS2 obtained by inserting a phosphoenolpyruvate carboxylase gene having been cloned and determined for its base sequence into a *Sall* site of a vector plasmid pBR322. pS2 has an ampicillin resistance gene as a drug resistance marker gene (Sabe, H. et al., *Gene*, 31, 279-283 (1984)). The nucleotide sequence of the phosphoenolpyruvate carboxylase gene contained in pS2 is the same as that contained in the aforementioned plasmid pT2.

pS2 DNA was treated at 75 °C for 2 hours with a hydroxylamine treating solution (20 µg/ml pS2 DNA, 0.05 M sodium phosphate (pH 6.0), 1 mM EDTA, 0.4 M hydroxylamine). Because of influence by pH on the hydroxylamine treatment, 80 µl of 1 M hydroxylamine · HCl and 1 mM EDTA solution having a pH adjusted to 6.0 with sodium hydroxide, 100 µl of 0.1 M sodium phosphate (pH 6.0) and 1 mM EDTA solution, and TE (10 mM Tris-HCl, 1 mM EDTA) buffer containing 2 µg of pS2 DNA were mixed, to finally provide 200 µl with water.

The aforementioned condition is a condition in which transformants has a survival ratio of 0.2 % based on a state before the treatment in an ampicillin-containing medium when *Escherichia coli* HB101 is transformed with pS2 after the treatment.

*Escherichia coli* HB101 was transformed with pS2 treated with hydroxylamine, which was spread on a solid plate medium containing ampicillin to obtain about 10000 colonies of transformants. They were suspended in a liquid medium, and spread on a solid plate medium containing any one of 3-bromopyruvate (3BP), aspartate-β-hydroxamate (AHX), aspartate-β-hydrazide (AHY) and DL-threo-β-hydroxyaspartate (pHA) as the analog compounds of aspartic acid at a concentration near a minimal inhibitory concentration to give 10<sup>3</sup> to 10<sup>5</sup> cells per one medium plate, and growing colonies were selected.

From 100 strains of analog compound resistant strains thus obtained, phosphoenolpyruvate carboxylase produced by each of them was partially purified in accordance with a method described in *The Journal of Biochemistry*, Vol. 67, No. 4 (1970), and inhibition of enzyme activity by the analog compounds was investigated. Measurement of the enzyme activity was performed in the same manner as described above.

Further, plasmids were isolated from bacterial strains producing mutant enzymes with activities not inhibited by the analog compounds, and were introduced into *Escherichia coli* PCR1 as a phosphoenolpyruvate carboxylase deficient strain (Sabe, H. et al., *Gene*, 31, 279-283 (1984)), to confirm production of the mutant enzymes.

Five transformants harboring mutant enzyme genes were thus obtained. As a result of determination of base sequences of these genes, 2 strains had the same mutation, and 4 kinds of mutant genes were obtained. The transformants harboring them were designated as AJ12907, AJ12908, AJ12909 and AJ12910, and were deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on August 3, 1993 under the deposition numbers of FERM P-13774, FERM P-13775, FERM P-13776 and FERM P-13777, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition numbers of FERM BP-4734, FERM BP-4735, FERM BP-4736, FERM BP-4737, respectively in this order. Further, the plasmids possessed by them were designated as pBP5, pHA19, pBP122 and pR6 respectively in this order. Mutations possessed by the phosphoenolpyruvate carboxylase genes contained in each of the plasmids are shown in Table 2. Numerical values in the table indicate nucleotide numbers or amino acid numbers in SEQ ID NO:1.

Table 2

Transformant	Plasmid	Mutation	Amino acid replacement associated with mutation
AJ12907	pBP5	2109 G→A	625 Glu→Lys
AJ12908	pHA19	901 G→A	222 Arg→His
		903 G→A	223 Glu→Lys
AJ12909	pBP122	1099 C→T	288 Ser→Phe
		1101 G→A	289 Glu→Lys
		1889 G→A	551 Met→Ile
		2646 G→A	804 Glu→Lys
AJ12910	pR6	2835 G→A	867 Ala→Thr

Incidentally, selection was performed for AJ12907 and AJ12909 in a medium containing 500 µg/ml of 3BP, for AJ12908 in a medium containing 1000 µg/ml of βHA, and for AJ12910 in a medium containing 500 µg/ml of AHY.

#### Example 2: mutant phosphoenolpyruvate carboxylase

Sensitivity to aspartic acid was investigated for phosphoenolpyruvate carboxylases produced by the aforementioned 4 transformants. These bacterial strains are deficient in the phosphoenolpyruvate carboxylase gene originating from the host, so that produced phosphoenolpyruvate carboxylase originates from the plasmid.

Sensitivity to aspartic acid was investigated in accordance with a known method (Yoshinaga, T., Izui, K. and Katsuki, H., *J. Biochem.*, 68, 747-750 (1970)). Namely, as a result of measurement of the enzyme activity produced by each of the transformants or *Escherichia coli* harboring pS2 in the presence of acetyl-coenzyme A known to affect the activity in an activity measurement system at a concentration of 0.1 mM or 1 mM, sensitivity to aspartic acid was measured as shown in Figs. 9 and 10.

According to the result, it is apparent that the wild type enzyme loses its activity when aspartic acid is at a high concentration, while the mutant phosphoenolpyruvate carboxylase of the present invention substantially continues to maintain its activity.

#### Example 3: fermentative production of L-threonine by *Escherichia coli* with introduced mutant phosphoenolpyruvate carboxylase

As threonine-producing bacteria of *Escherichia coli*, B-3996 strain (Japanese Patent Laid-open No. 3-501682 (PCT)) has the highest production ability among those known at present. Thus upon evaluation of the mutant phosphoenolpyruvate carboxylase, B-3996 was used as the host. This B-3996 strain has been deposited in Research Institute for Genetics and Industrial Microorganism Breeding under a registration number of RIA 1867. Further, pBP5 was selected as the mutant phosphoenolpyruvate carboxylase to be evaluated, which was subjected to an experiment.

The plasmid pBP5 having the mutant phosphoenolpyruvate carboxylase was introduced into *Escherichia coli* B-3996 in accordance with a method of Hanahan (*J. Mol. Biol.*, Vol. 106, p577 (1983)), and a transformant was isolated. As a control, *Escherichia coli* B-3996 was transformed in the same manner with pS2 as the plasmid to express the wild type phosphoenolpyruvate carboxylase gene.

When *Escherichia coli* B-3996 and the transformants therefrom were respectively inoculated in a 500 ml of Sakaguchi flask poured with 20 ml of a medium having a composition in Table 3, and cultivated at 37 °C for 40 hours to investigate a production amount of L-threonine, then results shown in Table 4 were obtained. Incidentally, the aforementioned medium was separated into two: glucose and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and the other components, and adjusted to have a pH of 7.0 with KOH followed by autoclaving at 115 °C for 10 minutes, and then, after mixing them, separately sterilized  $\text{CaCO}_3$  was added by 30 g/l.

Table 3

Component	Blending amount (g/l)
glucose	40
$(\text{NH}_4)_2\text{SO}_4$	16
$\text{KH}_2\text{PO}_4$	1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	0.01
yeast extract (Difco)	2
L-Met	0.5
$\text{CaCO}_3$	30

Table 4

Bacterial strain	Threonine production amount (g/l)
<u>Escherichia coli</u> B-3996	15.7
<u>Escherichia coli</u> B-3996/pS2	15.8
<u>Escherichia coli</u> B-3996/pBP5	16.8

As clarified from the result, Escherichia coli B-3996/pBP5 harboring the mutant enzyme expression plasmid having the DNA sequence of the present invention had an improved threonine-producing ability as compared with Escherichia coli B-3996/pS2 harboring the plasmid to express the wild type enzyme.

Example 4: fermentative production of L-alutamic acid by Escherichia coli with introduced mutant phosphoenolpyruvate carboxylase

As glutamic acid-producing bacteria of Escherichia coli, Escherichia coli AJ-12628 described in Japanese Patent Laid-open No. 4-11461 has the highest production ability among those known at present. Thus upon evaluation of the mutant phosphoenolpyruvate carboxylase, AJ-12628 was used as the host.

The AJ-12628 strain has been deposited in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology under a registration number of FERM BP-385. Further, pBP5 was selected as the mutant phosphoenolpyruvate carboxylase to be evaluated, which was subjected to an experiment.

The plasmid pBP5 having the mutant phosphoenolpyruvate carboxylase was introduced into Escherichia coli AJ-12628 in accordance with a method of Hanahan (J. Mol. Biol., Vol. 106, p577 (1983)), and a transformant was isolated. In the same manner, a transformant of Escherichia coli AJ-12628 with pS2 was isolated.

When Escherichia coli AJ-12628 and the transformants therefrom were respectively inoculated in a 500 ml of Sakaguchi flask poured with 20 ml of a medium having a composition in Table 5, and cultivated at 37 °C for 36 hours to investigate a production amount of L-glutamic acid, then results shown in Table 6 were obtained. Incidentally, the aforementioned medium was separated into two: glucose and  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and the other components, and adjusted to have a pH of 7.0 with KOH followed by autoclaving at 115 °C for 10 minutes, and then, after mixing them, separately sterilized  $\text{CaCO}_3$  was added by 30 g/l.

Table 5

Component	Blending amount (g/l)
glucose	40
$(\text{NH}_4)_2\text{SO}_4$	16
$\text{KH}_2\text{PO}_4$	1
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.01
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	0.01
yeast extract (Difco)	2
$\text{CaCO}_3$	30

Table 6

Bacterial strain	Glutamic acid production amount (g/l)
<u>Escherichia coli</u> AJ-12628	18.0
<u>Escherichia coli</u> AJ-12628/pS2	18.3
<u>Escherichia coli</u> AJ-12628/pBP5	19.6

As clarified from the result, Escherichia coli AJ-12628/pBP5 harboring the mutant enzyme expression plasmid having the DNA sequence of the present invention had an improved glutamate-producing ability as compared with Escherichia coli AJ-12628/pS2 harboring the plasmid to express the wild type enzyme.

Example 5: production of L-lysine by coryneform bacterium with introduced mutant phosphoenolpyruvate carboxylase

In order to introduce and express the mutant gene in a coryneform bacterium, a promoter originating from a bacterium belonging to the genus Brevibacterium was obtained, and was ligated with the mutant gene to prepare an expression type plasmid. Further, it was introduced into a bacterium belonging to the genus Brevibacterium to perform production of L-lysine.

(1) Acquisition of aspartokinase (AK) gene originating from bacterium belonging to the genus Brevibacterium

Chromosomal DNA was prepared according to an ordinary method from a Brevibacterium lactofermentum (Corynebacterium glutamicum) wild strain (ATCC 13869). An AK gene was amplified from the chromosomal DNA by PCR (polymerase chain reaction; see White, T. J. et al., Trends Genet., 5, 185 (1989)). For DNA primers used in the amplification, an oligonucleotide of 23 mer (SEQ ID NO:3) and an oligonucleotide of 21 mer (SEQ ID NO:4) were synthesized to amplify a region of about 1643 bp coding for the AK gene based on a sequence known in Corynebacterium glutamicum (see Molecular Microbiology (1991) 5 (5), 1197-1204, Mol. Gen. Genet. (1990) 224, 317-324).

The synthesis of DNA was performed in accordance with an ordinary phosphoramidite method (see Tetrahedron Letters (1981), 22, 1859) using a DNA synthesizer model 380B produced by Applied Biosystems Co. In the PCR reaction, DNA Thermal Cycler PJ2000 type produced by Takara Shuzo Co., Ltd. was used, and gene amplification was performed by using Taq DNA polymerase in accordance with a method designated by the manufacturer.

An amplified gene fragment of 1643 kb was confirmed by agarose gel electrophoresis, and then the fragment cut out from the gel was purified by an ordinary method, and was cleaved with restriction enzymes NruI (produced by Takara Shuzo Co., Ltd.) and EcoRI (produced by Takara Shuzo Co., Ltd.). pHSG399 (see Takeshita, S. et al.; Gene (1987), 61, 63-74) was used for a cloning vector for the gene fragment. pHSG399 was cleaved with a restriction enzyme SmaI (produced by Takara Shuzo Co., Ltd.) and a restriction enzyme EcoRI, and ligated with the amplified AK gene fragment.

Ligation of DNA was performed by a designated method by using a DNA ligation kit (produced by Takara Shuzo Co., Ltd.). In such a manner, a plasmid was manufactured in which pHSG399 was ligated with the AK gene fragment amplified from Brevibacterium chromosome. The plasmid having the AK gene originating from ATCC 13869 as the wild strain was designated as p399AKY.

(2) Determination of base sequence of AK gene of Brevibacterium lactofermentum

The AK plasmid, p399AKY was prepared, and the base sequence of the AK gene was determined. Determination of the base sequence was performed in accordance with the method of Sanger et al. (F. Sanger et al.: Proc. Natl. Acad. Sci. USA, 74, 5463 (1977) and so forth). Results are shown in SEQ ID NO:5 and SEQ ID NO:7. The DNA fragments have two open reading frames which correspond to  $\alpha$ -subunit and  $\beta$ -subunit of AK, respectively. In SEQ ID NO:5 and SEQ ID NO:7, amino acid sequences corresponding to each of the open reading frames are shown together with nucleotide sequences. Further, only the amino acid sequences corresponding to each of the open reading frames are shown in SEQ ID NO:6 and SEQ ID NO:8.

(3) Preparation of phosphoenolpyruvate carboxylase expression plasmid

Sall fragments of about 4.4 kb containing phosphoenolpyruvate carboxylase genes were extracted from pS2 as the plasmid having the wild type phosphoenolpyruvate carboxylase gene and pBP5 as the plasmid having the obtained

mutant phosphoenolpyruvate carboxylase gene, and inserted into a Sall site of a plasmid vector pHSG399 universally used for Escherichia coli. Manufactured plasmids were designated as pH2S for the wild type and as pHBP5 for the mutant.

In order to convert pH2S and pHBP5 into plasmids to express in Brevibacterium, a promoter and a replication origin of a plasmid for functioning in Brevibacterium were introduced. As the promoter, a gene fragment containing one from 1st NruI site to 207th ApaI site of the base sequence, which was postulated to be a promoter region of the cloned AK gene, was extracted from p399AKY, and inserted into an AvaI site located about 60 bp before the structural genes of pH2S and pHBP5 to allow the transcription direction to be in a regular direction.

Further, a gene fragment to enable autonomously replication of the plasmid in Brevibacterium, namely the replication origin of the plasmid was introduced into a site located on the vector. A gene fragment containing the replication origin of the plasmid was extracted from a vector pH4 for Brevibacterium (see paragraph No. 10 in Japanese Patent Laid-open No. 5-7491; Escherichia coli AJ12039 harboring the same plasmid is deposited in National Institute of Bio-science and Human Technology of Agency of Industrial Science and Technology, to which a deposition number of FERM P12215 is given), and restriction enzyme sites at both termini were modified into PstI sites by introduction of link-

This fragment was introduced into a PstI site in a vector portion of the plasmid added with the promoter derived from Brevibacterium. Constructed phosphoenolpyruvate carboxylase-expressing plasmids were designated as pH2SB for a wild type phosphoenolpyruvate carboxylase plasmid originating from p2S and as pHBP5B for a mutant phosphoenolpyruvate carboxylase plasmid originating from pBP5, respectively.

#### (4) Production of L-lysine by using phosphoenolpyruvate carboxylase expression type plasmid

Prepared pH2SB and pHBP5B were respectively introduced into AJ3463 as an L-lysine-producing bacterium of Brevibacterium lactofermentum (see Japanese Patent Publication No. 51-34477). For introduction of the gene, a transformation method employing electric pulse was used (see Japanese Patent Laid-open No. 2-207791). The host strain and transformants were cultivated with shaking for 72 hours at 31.5 °C in a lysine production medium having a composition in Table 7. The aforementioned medium was prepared such that those except for CaCO<sub>3</sub> among the components listed in the table were added to 1 l of water, and adjusted to have a pH of 8.0 with KOH followed by autoclaving at 115 °C for 15 minutes, and then CaCO<sub>3</sub> having been subjected to heat sterilization was further added. Accumulated amounts of L-lysine in the medium after cultivation are shown in Table 8.

Table 7

Component	Blending amount in 1 L
glucose	100 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	55 g
soybean concentrate*	35 ml
KH <sub>2</sub> PO <sub>4</sub>	1 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O	1 g
vitamin B1	20 g
biotin	5 g
nicotinic acid amide	5 mg
FeSO <sub>4</sub> · 7H <sub>2</sub> O	0.01 g
MnSO <sub>4</sub> · 5H <sub>2</sub> O	0.01 g
CaCO <sub>3</sub>	50g

\*: product of Ajinomoto Co., Ltd. (trade name: Mamenou)



Table 8

Bacterial strain	Lysine production amount (g/l)
<u>Brevibacterium lactofermentum</u> AJ3463	20.0
<u>Brevibacterium lactofermentum</u> AJ3463/pHS2B	22.0
<u>Brevibacterium lactofermentum</u> AJ3463/pHBP5B	25.0

As shown in the result, Brevibacterium lactofermentum AJ3463/pHBP5B harboring the mutant enzyme expression plasmid having the DNA sequence of the present invention had an improved lysine-producing ability as compared with Brevibacterium lactofermentum AJ3463/pHS2B harboring the plasmid to express the wild type enzyme.

Example 6: another example of mutant phosphoenolpyruvate carboxylase of the present invention and its gene

(1) Preparation of mutant phosphoenolpyruvate carboxylase gene

Upon preparation of DNA coding for a mutant phosphoenolpyruvate carboxylase, a phosphoenolpyruvate carboxylase gene cloned in a plasmid pT2 was used as a material.

A host, which is allowed to harbor the plasmid pT2, is preferably deficient in phosphoenolpyruvate carboxylase gene in order to detect only the activity of phosphoenolpyruvate carboxylase originating from the plasmid. Escherichia coli F15 (Hfr, recA1, met,  $\Delta$ (ppc-argECBH), Tn10) was used as such a deficient strain. Escherichia coli AJ-12873, which is allowed to harbor pT2 in F15 strain, is deposited as FERM P-13752 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on July 15, 1993, transferred from the original deposition to international deposition based on Budapest Treaty on

July 11, 1994 and has been deposited as deposition number of FERM BP-4732. In addition, an entire base sequence of pT2 is shown in SEQUENCE ID NO:1.

In order to replace a codon of 438th arginine of the phosphoenolpyruvate carboxylase into a codon of cysteine by using pT2, the Overlapping Extension method (Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R., Gene, 77, 51-59 (1989)) utilizing the PCR (Polymerase Chain Reaction) method was used.

Incidentally, the PCR method is a method in which an amplification cycle comprising thermal denaturation of double strand DNA into single strand DNA, annealing of oligonucleotide primers corresponding to sequences at both ends of a site aimed to be amplified and the aforementioned thermally denatured DNA, and polymerase reaction using the aforementioned oligonucleotides as primers is repeated, thereby the aforementioned DNA sequence is amplified in a manner of an exponential function.

A region subjected to site specific mutation by the PCR method is shown in Fig. 11. The primers used in the present invention were 4 species of a primer c (SEQUENCE ID NO:11, corresponding to base Nos. 1535-1554 in SEQUENCE ID NO:1) having a sequence in the vicinity of the codon of 438th arginine, a primer b (SEQUENCE ID NO:10) having a sequence complement to the primer c, a primer a (SEQUENCE ID NO:9, corresponding to base Nos. 1185-1200 in SEQUENCE ID NO:1) having a sequence upstream therefrom, and a primer d (SEQUENCE ID NO:12, corresponding to base Nos. 2327-2342 in SEQUENCE ID NO:1) having a sequence complement to a downstream sequence.

In the primer b and the primer c, the codon (CGT) of 438th arginine was replaced with a codon (TGT) of cysteine. This replacement may use TGC which is another codon of cysteine. Further, C of the third letter of a codon (AAC) of 435th asparagine was replaced with T, and hence an EcoRI site was internally introduced with no replacement of amino acid, so that a mutant plasmid could be selected by using it as an index. However, this mutation is not essential to the present invention.

When the PCR reaction was performed by using pT2 DNA as a template and the primer a and the primer b as the primers, a fragment from the upstream of the mutation site to the mutation site (AB fragment in Fig. 11) was amplified. Further, when the PCR reaction was performed by using the primer c and the primer d, a fragment downstream from the mutation site (CD fragment in Fig. 11) was amplified. When each of the amplified products (AB, CD) was annealed again after thermal denaturation to perform a polymerase reaction, they were ligated to obtain a fragment (AD fragment in Fig. 11). Incidentally, the PCR reaction was performed by repeating 30 cycles of each comprising heating at 94 °C for 1 minute followed by denaturation (94 °C, 1.5 minutes), annealing (50 °C, 2 minutes), and elongation reaction by polymerase (72 °C, 3.5 minutes). In addition, reaction compositions are shown in Table 9.

Table 9

Composition (( ): final conc.)	PCR fragment		
	AB	CD	AD
H <sub>2</sub> O	53.5	53.5	53.5
10-fold reaction buffer	10	10	10
mixture of 1.25 mM dNTP	16	16	16
20 $\mu$ M primer a (1 $\mu$ M)	5	-	5
20 $\mu$ M primer b (1 $\mu$ M)	5	-	-
20 $\mu$ M primer c (1 $\mu$ M)	-	5	-
20 $\mu$ M primer d (1 $\mu$ M)	-	5	5
10 $\mu$ g/ $\mu$ l pT2 (0.1 $\mu$ g)	10	10	-
PCR fragment AB*	-	-	5
PCR fragment CD*	-	-	5
2.5 U/ $\mu$ l <u>Tag</u> polymerase	0.5	0.5	0.5
total amount	100 $\mu$ l	100 $\mu$ l	100 $\mu$ l

\*: PCR fragments AB and CD were prepared, after the PCR reaction, by recovering 10  $\mu$ l thereof from polyacrylamide gel, and dissolving it in 5  $\mu$ l of TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)).

In the AD fragment obtained as described above, a BssHII site (1231-1236 in SEQ ID NO:1) at the upstream side and a SplI site (2249-2254 in SEQ ID NO:1) at the downstream side were present, so that complete digestion was performed with these enzymes to make replacement for a corresponding region of the plasmid pT2 (Fig. 11).

### (2) Selection of inhibition-desensitized phosphoenolpyruvate carboxylase

Escherichia coli was transformed with a plasmid obtained as described above, and a transformed strain was cultivated to recover the plasmid to select one cleaved by EcoRI. With respect to selected DNA, a base sequence of the region amplified by the aforementioned PCR method was determined by the dideoxy method to confirm that base replacement as exactly aimed was introduced. This plasmid was designated as pT2R438C. A strain (AJ12874) obtained by introducing this plasmid into the aforementioned Escherichia coli F15 has been deposited as FERM P-13753 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology (1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan; zip code 305) on July 15, 1993, transferred from the original deposition to international deposition based on Budapest Treaty on July 11, 1994 and has been deposited as deposition number of FERM BP-4733.

The base sequence of pT2R438C is a sequence in which 1541th and 1550th nucleotides are replaced from C to T respectively in SEQ ID NO:1.

### (3) Confirmation of desensitization of inhibition of mutant phosphoenolpyruvate carboxylase by aspartic acid

Sensitivity to aspartic acid was investigated for phosphoenolpyruvate carboxylase produced by the aforementioned Escherichia coli AJ12874 harboring pT2R438C. Incidentally, as described above, because the Escherichia coli F15 is deficient in phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase produced by AJ12874 originates from the plasmid.

Sensitivity to aspartic acid was investigated in accordance with a known method (Yoshinaga, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (1970)). Namely, as a result of measurement of the enzyme activity in the presence of acetyl-coenzyme A known to affect the activity in an activity measurement system at a concentration of 1 mM or 2 mM, sensitivity to aspartic acid was measured as shown in Fig. 12.

It is apparent that the wild type enzyme substantially loses its activity when aspartic acid is at a high concentration, while the mutant phosphoenolpyruvate carboxylase of the present invention continues to maintain its activity.

#### (4) Preparation of mutant phosphoenolpyruvate carboxylase gene (II)

In order to replace a codon of 620th lysine with a codon of serine in the phosphoenolpyruvate carboxylase gene carried on the plasmid pT2, the Overlapping Extension method (Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. and Pease, L.R., Gene, 77, 51-59 (1989)) utilizing the PCR (Polymerase Chain Reaction) method was used. Concrete procedures were in accordance with the method described in (1). A plasmid carrying a mutant gene constructed with aimed replacement was designated as pT2K620S. Further, an obtained mutant enzyme was designated as K620S mutant enzyme.

#### (5) Confirmation of desensitization of inhibition by aspartic acid concerning mutant phosphoenolpyruvate carboxylase.

With respect to the phosphoenolpyruvic carboxylase produced by a transformant obtained by introducing the plasmid pT2K620S into the aforementioned Escherichia coli F15, sensitivity to aspartic acid was investigated. Incidentally, as described above, since the Escherichia coli F15 lacks phosphoenolpyruvate carboxylase, any phosphoenolpyruvate carboxylase produced by the transformant originates from the plasmid.

Sensitivity to aspartic acid was investigated in accordance with a known method (Yoshinaga, T., Izui, K. and Katsuki, H., J. Biochem., 68, 747-750 (1970)). Namely, as a result of measurement of the enzyme activity in the presence of acetyl-coenzyme A known to affect the activity in an activity measurement system at a concentration of 1 mM or 2 mM, sensitivity to aspartic acid was measured as shown in Fig. 13.

It is apparent that the wild enzyme substantially loses its activity when aspartic acid is at a high concentration, while the type phosphoenolpyruvate carboxylase of the present invention continues to maintain its activity.

In Fig. 13, sensitivity to aspartic acid is also depicted for a mutant phosphoenolpyruvate carboxylase in which 650th lysine is replaced with serine (K650A mutant enzyme), and for a mutant phosphoenolpyruvate carboxylase in which 491th lysine is replaced with serine (K491A mutant enzyme). In the case of these mutant enzymes, inhibition by aspartic acid was not desensitized.

#### INDUSTRIAL APPLICABILITY

The DNA sequence of the present invention codes for the mutant phosphoenolpyruvate carboxylase, and the microorganism harboring this DNA sequence produces the aforementioned enzyme.

The mutant phosphoenolpyruvate carboxylase of the present invention does not substantially undergo activity inhibition by aspartic acid, so that it can be utilized for fermentative production of amino acids subjected to regulation of biosynthesis by aspartic acid and the like.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Ajinomoto Co. Inc.

(A) NAME:

(B) STREET: 15-1, Kyobashi 1-chome, Chuo-ku

(C) CITY: Tokyo

(D) STATE OR PROVINCE:

(E) COUNTRY: Japan

(F) POSTAL CODE: 104

(ii) TITLE OF INVENTION: Mutant Phosphoenolpyruvate Carboxylase, Its  
gene, and Production Method of Amino Acid

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(v) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

## (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5186

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: circular

(ii) MOLECULAR TYPE: other..genomic DNA and vector DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Escherichia coli

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 237..2888

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TCGACCGGCG	ATTTTAAAC	ATTTCATAA	GTTACGCTTA	TTTAAAGCGT	CGTGAATTTA	60
ATGACGTAAA	TTCTGCTAT	TTATTCGTTT	GCTGAAGOGA	TTTCGCAGCA	TTTGACGTCA	120
CCGCTTTTAC	GTGGCTTTAT	AAAAGACGAC	GAAAAGCAAA	GCCCGAGCAT	ATTGCGGCA	180

	ATGCGACGTG AAGGATACAG GGCTATCAAA CGATAAGATG GGGTGTCTGG GGTAAT	236
	ATG AAC GAA CAA TAT TCC GCA TTG CGT AGT AAT GTC AGT ATG CTC GGC	284
5	Met Asn Glu Gln Tyr Ser Ala Leu Arg Ser Asn Val Ser Met Leu Gly	
	1 5 10 15	
	AAA GTG CTG GGA GAA ACC ATC AAG GAT GCG TTG GGA GAA CAC ATT CTT	332
	Lys Val Leu Gly Glu Thr Ile Lys Asp Ala Leu Gly Glu His Ile Leu	
	20 25 30	
10	GAA CGC GTA GAA ACT ATC CGT AAG TTG TCG AAA TCT TCA CGC GCT GGC	380
	Glu Arg Val Glu Thr Ile Arg Lys Leu Ser Lys Ser Ser Arg Ala Gly	
	35 40 45	
	AAT GAT GCT AAC CGC CAG GAG TTG CTC ACC ACC TTA CAA AAT TTG TCG	428
15	Asn Asp Ala Asn Arg Gln Glu Leu Leu Thr Thr Leu Gln Asn Leu Ser	
	50 55 60	
	AAC GAC GAG CTG CTG CCC GTT GCG CGT GCG TTT AGT CAG TTC CTG AAC	476
	Asn Asp Glu Leu Leu Pro Val Ala Arg Ala Phe Ser Gln Phe Leu Asn	
	65 70 75 80	
20	CTG GCC AAC ACC GCC GAG CAA TAC CAC AGC ATT TCG CCG AAA GGC GAA	524
	Leu Ala Asn Thr Ala Glu Gln Tyr His Ser Ile Ser Pro Lys Gly Glu	
	85 90 95	
	GCT GCC AGC AAC CCG GAA GTG ATC GGC CGC ACC CTG CGT AAA CTG AAA	572
25	Ala Ala Ser Asn Pro Glu Val Ile Ala Arg Thr Leu Arg Lys Leu Lys	
	100 105 110	
	AAC CAG CCG GAA CTG AGC GAA GAC ACC ATC AAA AAA GCA GTG GAA TCG	620
	Asn Gln Pro Glu Leu Ser Glu Asp Thr Ile Lys Lys Ala Val Glu Ser	
	115 120 125	
30	CTG TCG CTG GAA CTG GTC CTC ACG GCT CAC CCA ACC GAA ATT ACC CGT	668
	Leu Ser Leu Glu Leu Val Leu Thr Ala His Pro Thr Glu Ile Thr Arg	
	130 135 140	
	CGT ACA CTG ATC CAC AAA ATG GTG GAA GTG AAC GCC TGT TTA AAA CAG	716
35	Arg Thr Leu Ile His Lys Met Val Glu Val Asn Ala Cys Leu Lys Gln	
	145 150 155 160	
	CTC GAT AAC AAA GAT ATC GCT GAC TAC GAA CAC AAC CAG CTG ATG CGT	764
	Leu Asp Asn Lys Asp Ile Ala Asp Tyr Glu His Asn Gln Leu Met Arg	
	165 170 175	
40	CGC CTG CGC CAG TTG ATC GCC CAG TCA TGG CAT ACC GAT GAA ATC CGT	812
	Arg Leu Arg Gln Leu Ile Ala Gln Ser Trp His Thr Asp Glu Ile Arg	
	180 185 190	
	AAG CTG CGT CCA AGC CCG GTA GAT GAA GCC AAA TGG GGC TTT GCC GTA	860
45	Lys Leu Arg Pro Ser Pro Val Asp Glu Ala Lys Trp Gly Phe Ala Val	
	195 200 205	
	GTG GAA AAC AGC CTG TGG CAA GGC GTA CCA AAT TAC CTG CGC GAA CTG	908
	Val Glu Asn Ser Leu Trp Gln Gly Val Pro Asn Tyr Leu Arg Glu Leu	
	210 215 220	
50		
55		

	AAC GAA CAA CTG GAA GAG AAC CTC GGC TAC AAA CTG CCC GTC GAA TTT	956
	Asn Glu Gln Leu Glu Glu Asn Leu Gly Tyr Lys Leu Pro Val Glu Phe	
	225 230 235 240	
5	GTT CCG GTC CGT TTT ACT TCG TGG ATG GGC GGC GAC CGC GAC GGC AAC	1004
	Val Pro Val Arg Phe Thr Ser Trp Met Gly Gly Asp Arg Asp Gly Asn	
	245 250 255	
	COG AAC GTC ACT GGC GAT ATC ACC CGC CAC GTC CTG CTA CTC AGC CGC	1052
10	Pro Asn Val Thr Ala Asp Ile Thr Arg His Val Leu Leu Leu Ser Arg	
	260 265 270	
	TGG AAA GGC ACC GAT TTG TTC CTG AAA GAT ATT CAG GTG CTG GTT TCT	1100
	Trp Lys Ala Thr Asp Leu Phe Leu Lys Asp Ile Gln Val Leu Val Ser	
	275 280 285	
15	GAA CTG TCG ATG GTT GAA GCG ACC CCT GAA CTG CTG GCG CTG GTT GGC	1148
	Glu Leu Ser Met Val Glu Ala Thr Pro Glu Leu Leu Ala Leu Val Gly	
	290 295 300	
	GAA GAA GGT GCC GCA GAA CCG TAT CCG TAT CTG ATG AAA AAC CTG CGT	1196
20	Glu Glu Gly Ala Ala Glu Pro Tyr Arg Tyr Leu Met Lys Asn Leu Arg	
	305 310 315 320	
	TCT CCG CTG ATG GCG ACA CAG GCA TGG CTG GAA GCG CCG CTG AAA GGC	1244
	Ser Arg Leu Met Ala Thr Gln Ala Trp Leu Glu Ala Arg Leu Lys Gly	
	325 330 335	
25	GAA GAA CTG CCA AAA CCA GAA GGC CTG CTG ACA CAA AAC GAA GAA CTG	1292
	Glu Glu Leu Pro Lys Pro Glu Gly Leu Leu Thr Gln Asn Glu Glu Leu	
	340 345 350	
	TGG GAA CCG CTC TAC GCT TGC TAC CAG TCA CTT CAG GCG TGT GGC ATG	1340
30	Trp Glu Pro Leu Tyr Ala Cys Tyr Gln Ser Leu Gln Ala Cys Gly Met	
	355 360 365	
	GGT ATT ATC GCC AAC GGC GAT CTG CTC GAC ACC CTG CCG CCG GTG AAA	1388
	Gly Ile Ile Ala Asn Gly Asp Leu Leu Asp Thr Leu Arg Arg Val Lys	
	370 375 380	
35	TGT TTC GGC GTA CCG CTG GTC CGT ATT GAT ATC CGT CAG GAG AGC ACG	1436
	Cys Phe Gly Val Pro Leu Val Arg Ile Asp Ile Arg Gln Glu Ser Thr	
	385 390 395 400	
	CGT CAT ACC GAA GCG CTG GGC GAG CTG ACC CCG TAC CTC GGT ATC GGC	1484
40	Arg His Thr Glu Ala Leu Gly Glu Leu Thr Arg Tyr Leu Gly Ile Gly	
	405 410 415	
	GAC TAC GAA AGC TGG TCA GAG GCC GAC AAA CAG GCG TTC CTG ATC CCG	1532
	Asp Tyr Glu Ser Trp Ser Glu Ala Asp Lys Gln Ala Phe Leu Ile Arg	
	420 425 430	
45	GAA CTG AAC TCC AAA CGT CCG CTT CTG CCG CCG AAC TGG CAA CCA AGC	1580
	Glu Leu Asn Ser Lys Arg Pro Leu Leu Pro Arg Asn Trp Gln Pro Ser	
	435 440 445	
	GCC GAA ACG CCG GAA GTG CTC GAT ACC TGC CAG GTG ATT GCC GAA GCA	1628
50	Ala Glu Thr Arg Glu Val Leu Asp Thr Cys Gln Val Ile Ala Glu Ala	
	450 455 460	

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	COG	CAA	GGC	TCC	ATT	GCC	GCC	TAC	GTG	ATC	TCG	ATG	GCG	AAA	ACG	CCG	1676
	Pro	Gln	Gly	Ser	Ile	Ala	Ala	Tyr	Val	Ile	Ser	Met	Ala	Lys	Thr	Pro	
5	465					470					475					480	
	TOC	GAC	GTA	CTG	GCT	GTC	CAC	CTG	CTG	CTG	AAA	GAA	GCG	GGT	ATC	GGG	1724
	Ser	Asp	Val	Leu	Ala	Val	His	Leu	Leu	Leu	Lys	Glu	Ala	Gly	Ile	Gly	
					485					490					495		
10	TTT	GCG	ATG	CCG	GTT	GCT	CCG	CTG	TTT	GAA	ACC	CTC	GAT	GAT	CTG	AAC	1772
	Phe	Ala	Met	Pro	Val	Ala	Pro	Leu	Phe	Glu	Thr	Leu	Asp	Asp	Leu	Asn	
				500					505						510		
	AAC	GCC	AAC	GAT	GTC	ATG	ACC	CAG	CTG	CTC	AAT	ATT	GAC	TGG	TAT	CGT	1820
	Asn	Ala	Asn	Asp	Val	Met	Thr	Gln	Leu	Leu	Asn	Ile	Asp	Trp	Tyr	Arg	
				515				520					525				
15	GGC	CTG	ATT	CAG	GGC	AAA	CAG	ATG	GTG	ATG	ATT	GGC	TAT	TOC	GAC	TCA	1868
	Gly	Leu	Ile	Gln	Gly	Lys	Gln	Met	Val	Met	Ile	Gly	Tyr	Ser	Asp	Ser	
				530				535					540				
20	GCA	AAA	GAT	GCG	GGA	GTG	ATG	GCA	GCT	TOC	TGG	GCG	CAA	TAT	CAG	GCA	1916
	Ala	Lys	Asp	Ala	Gly	Val	Met	Ala	Ala	Ser	Trp	Ala	Gln	Tyr	Gln	Ala	
	545					550					555					560	
	CAG	GAT	GCA	TTA	ATC	AAA	ACC	TGC	GAA	AAA	GCG	GGT	ATT	GAG	CTG	ACG	1964
	Gln	Asp	Ala	Leu	Ile	Lys	Thr	Cys	Glu	Lys	Ala	Gly	Ile	Glu	Leu	Thr	
					565					570					575		
25	TTG	TTC	CAC	GGT	OGC	GGC	GGT	TOC	ATT	GGT	OGC	GGC	GGC	GCA	OCT	GCT	2012
	Leu	Phe	His	Gly	Arg	Gly	Gly	Ser	Ile	Gly	Arg	Gly	Gly	Ala	Pro	Ala	
				580					585						590		
30	CAT	GCG	GCG	CTG	CTG	TCA	CAA	COG	OCA	GGA	AGC	CTG	AAA	GGC	GGC	CTG	2060
	His	Ala	Ala	Leu	Leu	Ser	Gln	Pro	Pro	Gly	Ser	Leu	Lys	Gly	Gly	Leu	
				595				600						605			
	OGC	GTA	ACC	GAA	CAG	GGC	GAG	ATG	ATC	OGC	TTT	AAA	TAT	GGT	CTG	CCA	2108
	Arg	Val	Thr	Glu	Gln	Gly	Glu	Met	Ile	Arg	Phe	Lys	Tyr	Gly	Leu	Pro	
				610				615					620				
35	GAA	ATC	ACC	GTC	AGC	AGC	CTG	TCG	CTT	TAT	ACC	GGG	GCG	ATT	CTG	GAA	2156
	Glu	Ile	Thr	Val	Ser	Ser	Leu	Ser	Leu	Tyr	Thr	Gly	Ala	Ile	Leu	Glu	
						630						635				640	
	GCC	AAC	CTG	CTG	CCA	COG	GAG	COG	AAA	GAG	AGC	TGG	CGT	OGC	ATT		2204
40	Ala	Asn	Leu	Leu	Pro	Pro	Glu	Pro	Lys	Glu	Ser	Trp	Arg	Arg	Ile		
					645					650					655		
	ATG	GAT	GAA	CTG	TCA	GTC	ATC	TOC	TGC	GAT	GTC	TAC	OGC	GGC	TAC	GTA	2252
	Met	Asp	Glu	Leu	Ser	Val	Ile	Ser	Cys	Asp	Val	Tyr	Arg	Gly	Tyr	Val	
					660					665					670		
45	CGT	GAA	AAC	AAA	GAT	TTT	GTG	CCT	TAC	TTC	OGC	TOC	GCT	ACG	COG	GAA	2300
	Arg	Glu	Asn	Lys	Asp	Phe	Val	Pro	Tyr	Phe	Arg	Ser	Ala	Thr	Pro	Glu	
				675				680							685		
	CAA	GAA	CTG	GGC	AAA	CTG	COG	TTG	GGT	TCA	CGT	COG	GCG	AAA	CGT	CGC	2348
50	Gln	Glu	Leu	Gly	Lys	Leu	Pro	Leu	Gly	Ser	Arg	Pro	Ala	Lys	Arg	Arg	
				690				695							700		

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	OCA	ACC	GGC	GGC	GTC	GAG	TCA	CTA	CGC	GCC	ATT	CCG	TGG	ATC	TTC	GCC	2396
	Pro	Thr	Gly	Gly	Val	Glu	Ser	Leu	Arg	Ala	Ile	Pro	Trp	Ile	Phe	Ala	
5	705					710				715					720		
	TGG	ACG	CAA	AAC	OGT	CTG	ATG	CTC	CCC	GCC	TGG	CTG	GGT	GCA	GGT	ACG	2444
	Trp	Thr	Gln	Asn	Arg	Leu	Met	Leu	Pro	Ala	Trp	Leu	Gly	Ala	Gly	Thr	
					725					730					735		
10	GCG	CTG	CAA	AAA	GTG	GTC	GAA	GAC	GGC	AAA	CAG	AGC	GAG	CTG	GAG	GCT	2492
	Ala	Leu	Gln	Lys	Val	Val	Glu	Asp	Gly	Lys	Gln	Ser	Glu	Leu	Glu	Ala	
					740					745					750		
	ATG	TGC	CGC	GAT	TGG	OCA	TTC	TTC	TCG	ACG	OGT	CTC	GGC	ATG	CTG	GAG	2540
	Met	Cys	Arg	Asp	Trp	Pro	Phe	Phe	Ser	Thr	Arg	Leu	Gly	Met	Leu	Glu	
15					755					760					765		
	ATG	GTC	TTC	GCC	AAA	GCA	GAC	CTG	TGG	CTG	GCG	GAA	TAC	TAT	GAC	CAA	2588
	Met	Val	Phe	Ala	Lys	Ala	Asp	Leu	Trp	Leu	Ala	Glu	Tyr	Tyr	Asp	Gln	
					770					775					780		
20	CGC	CTG	GTA	GAC	AAA	GCA	CTG	TGG	CCG	TTA	GGT	AAA	GAG	TTA	CGC	AAC	2636
	Arg	Leu	Val	Asp	Lys	Ala	Leu	Trp	Pro	Leu	Gly	Lys	Glu	Leu	Arg	Asn	
						785									790		
	CTG	CAA	GAA	GAA	GAC	ATC	AAA	GTG	GTG	CTG	GCG	ATT	GCC	AAC	GAT	TOC	2684
	Leu	Gln	Glu	Glu	Asp	Ile	Lys	Val	Val	Leu	Ala	Ile	Ala	Asn	Asp	Ser	
25					805					810					815		
	CAT	CTG	ATG	GCC	GAT	CTG	CCG	TGG	ATT	GCA	GAG	TCT	ATT	CAG	CTA	CGG	2732
	His	Leu	Met	Ala	Asp	Leu	Pro	Trp	Ile	Ala	Glu	Ser	Ile	Gln	Leu	Arg	
					820					825					830		
30	AAT	ATT	TAC	ACC	GAC	CCG	CTG	AAC	GTA	TTG	CAG	GCC	GAG	TTG	CTG	CAC	2780
	Asn	Ile	Tyr	Thr	Asp	Pro	Leu	Asn	Val	Leu	Gln	Ala	Glu	Leu	Leu	His	
					835					840					845		
	CGC	TOC	CGC	CAG	GCA	GAA	AAA	GAA	GGC	CAG	GAA	CCG	GAT	OCT	CGC	GTC	2828
	Arg	Ser	Arg	Gln	Ala	Glu	Lys	Glu	Gly	Gln	Glu	Pro	Asp	Pro	Arg	Val	
35					850					855					860		
	GAA	CAA	GCG	TTA	ATG	GTC	ACT	ATT	GCC	GGG	ATT	GCG	GCA	GGT	ATG	CGT	2876
	Glu	Gln	Ala	Leu	Met	Val	Thr	Ile	Ala	Gly	Ile	Ala	Ala	Gly	Met	Arg	
					865					870					875		
	AAT	ACC	GGC	TAATCTTCT	CTTCTGCAAA	CCCTCGTGCT	TTTGCGCGAG										2925
40	Asn	Thr	Gly														
	GGTTTTCTGA	AATACITCTG	TTCTAACACC	CTCGTTTTCA	ATATATTTCT	GTCTGCATTT											2985
	TATTCAAATT	CTGAATATAC	CTTCAGATAT	CCTTAAGGGC	CTCGTGATAC	GOCTATTTTT											3045
	ATAGGTAAAT	GTCATGATAA	TAATGGTTTC	TTAGACGTCA	GGTGGCACTT	TTGGGGGAAA											3105
	TGTGCGGGGA	ACCCCTATTT	GTATTATTTT	CTAAATACAT	TCAAATATGT	ATCOGCTCAT											3165
45	GAGACAATAA	COCTGATAAA	TGCTTCAATA	ATATTGAAAA	AGGAAGAGTA	TGAGTATTCA											3225
	ACATTTTOGT	GTCGOCCTTA	TTCCCTTTTT	TGCGGCATTT	TGCTTCTCTG	TTTTTGCTCA											3285
	CCAGAAACG	CTGGTGAAAG	TAAAAGATGC	TGAAGATCAG	TTGGGTGCAC	GAGTGGGTTA											3345
	CATOGAACTG	GATCTCAACA	GCGGTAAGAT	CCTTGAGAGT	TTTGGCCCCG	AAGAACTTTT											3405
50	TOCAATGATG	AGCACITTTTA	AAGTCTGTCT	ATGTGGGOGG	GTATTATCCC	GTATTGAOGC											3465
	CGGGCAAGAG	CAACTCGGTC	GCGCATACA	CTATTCTCAG	AATGACTTGG	TTGAGTACTC											3525



	ACCAAGTCACA	GAAAAGCATC	TTACGGATGG	CATGACAGTA	AGAGAATTAT	GCAGTGCTGC	3585
	CATAACCATG	AGTGATAACA	CTGCGGCCAA	CTTACTTCTG	ACAACGATCG	GAGGACOGAA	3645
5	GGAGCTAACC	GCTTTTITGC	ACAACATGGG	GGATCATGTA	ACTCGCCTTG	ATCGTTGGGA	3705
	ACCGGAGCTG	AATGAAGCCA	TACCAACGA	CGAGCGTGAC	ACCACGATGC	CTGTAGCAAT	3765
	GGCAACAACG	TTGCGCAAAC	TATTAACCTG	CGAACTACTT	ACTCTAGCTT	CCCGGCAACA	3825
	ATTAATAGAC	TGGATGGAGG	CGGATAAAGT	TGCAGGACCA	CTTCTGCGCT	CGGCCCTTCC	3885
	GGCTGGCTGG	TTTATTGCTG	ATAAATCTGG	AGCCGGTGAG	CGTGGGTCTC	GCGGTATCAT	3945
10	TGCAGCACTG	GGGOCAGATG	GTAAGCCCTC	CCGTATCGTA	GTTATCTACA	CGACGGGGAG	4005
	TCAGGCAACT	ATGGATGAAC	GAAATAGACA	GATCGCTGAG	ATAGGTGCCT	CACTGATTAA	4065
	GCATTGGTAA	CTGTCAGACC	AAGTTTACTC	ATATATACTT	TAGATTGATT	TAAAACCTCA	4125
	TTTTTAATTT	AAAAGGATCT	AGGTGAAGAT	CCTTTTTTGAT	AATCTCATGA	CCAAAATCCC	4185
	TTAACGTGAG	TTTTGCTTCC	ACTGAGCGTC	AGACCCCGTA	GAAAAGATCA	AAGGATCTTC	4245
15	TTGAGATCCT	TTTTTTCTGC	GCGTAATCTG	CTGCTTGCAA	ACAAAAAAC	CACCGCTACC	4305
	AGCGGTGGTT	TGTTTGCCGG	ATCAAGAGCT	ACCAACTCTT	TTTCOGAAGG	TAAGTGGCTT	4365
	CAGCAGAGCG	CAGATAACAA	ATACTGTCTT	TCTAGTGTAG	CCGTAGTTAG	GCCACCACTT	4425
	CAAGAACTCT	GTAGCACCGC	CTACATACTT	CGCTCTGCTA	ATCCTGTTAC	CAGTGGCTGC	4485
20	TGCCAGTGGC	GATAAGTGGT	GTCCTAACGG	GTTGGACTCA	AGACGATAGT	TACCGGATAA	4545
	GGGCGAGCGG	TGCGGCTGAA	CGGGGGTTC	GTGCACACAG	CCAGCCTTGG	AGCGAACGAC	4605
	CTACACCGAA	CTGAGATAAC	TACAGCGTGA	GCATTGAGAA	AGCGCCACGC	TTCCCGAAGG	4665
	GAGAAAGGCG	GACAGGTATC	CGGTAAGCGG	CAGGGTCGGA	ACAGGAGAGC	GCAOGAGGGA	4725
	GCTTCCAGGG	GGAAACGCTT	GGTATCTTTA	TAGTCTGTGC	GGGTTTCGCC	ACCTCTGACT	4785
25	TGAGCGTCTA	TTTTTGTGAT	GCTGCTCAGG	GGGGCGGAGC	CTATGGAAAA	ACGCCAGCAA	4845
	CGCGGCTTTT	TTACGGTTCC	TGGCCTTTTG	CTGGCCTTTT	GCTCACATGT	TCTTTCTGTC	4905
	GTTATCCCTT	GATTCGTGTT	ATAACCGTAT	TACCGCCTTT	GAGTGAGCTG	ATACCGCTCG	4965
	CCGCAACCGA	AGACCGAGC	GCAGCGAGTC	AGTGAGCGAG	GAAGCGGAAG	AGCGCCCAAT	5025
30	ACGCAAAACG	CCTCTCCCCG	CGCGTTGGCC	GATTCAATTAA	TGCAGAAGGG	TTGGTTTGGC	5085
	CATTACAGAT	TCTCCGCAAG	AATTGATTGG	CTCCAATTCT	TGGAGTGGTG	AATCCGTTAG	5145
	CGAGGTGCCG	CGGCCTTCCA	TTCAGGTCTA	GGTGGCCCGG	G		5186

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 883 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asn Glu Gln Tyr Ser Ala Leu Arg Ser Asn Val Ser Met Leu Gly  
 1 5 10 15  
 Lys Val Leu Gly Glu Thr Ile Lys Asp Ala Leu Gly Glu His Ile Leu  
 20 25 30  
 Glu Arg Val Glu Thr Ile Arg Lys Leu Ser Lys Ser Ser Arg Ala Gly  
 35 40 45  
 Asn Asp Ala Asn Arg Gln Glu Leu Leu Thr Thr Leu Gln Asn Leu Ser  
 50 55 60

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5 Asn Asp Glu Leu Leu Pro Val Ala Arg Ala Phe Ser Gln Phe Leu Asn  
 65 70 75 80  
 Leu Ala Asn Thr Ala Glu Gln Tyr His Ser Ile Ser Pro Lys Gly Glu  
 85 90 95  
 Ala Ala Ser Asn Pro Glu Val Ile Ala Arg Thr Leu Arg Lys Leu Lys  
 100 105 110  
 10 Asn Gln Pro Glu Leu Ser Glu Asp Thr Ile Lys Lys Ala Val Glu Ser  
 115 120 125  
 Leu Ser Leu Glu Leu Val Leu Thr Ala His Pro Thr Glu Ile Thr Arg  
 130 135 140  
 Arg Thr Leu Ile His Lys Met Val Glu Val Asn Ala Cys Leu Lys Gln  
 145 150 155 160  
 15 Leu Asp Asn Lys Asp Ile Ala Asp Tyr Glu His Asn Gln Leu Met Arg  
 165 170 175  
 Arg Leu Arg Gln Leu Ile Ala Gln Ser Trp His Thr Asp Glu Ile Arg  
 180 185 190  
 20 Lys Leu Arg Pro Ser Pro Val Asp Glu Ala Lys Trp Gly Phe Ala Val  
 195 200 205  
 Val Glu Asn Ser Leu Trp Gln Gly Val Pro Asn Tyr Leu Arg Glu Leu  
 210 215 220  
 25 Asn Glu Gln Leu Glu Glu Asn Leu Gly Tyr Lys Leu Pro Val Glu Phe  
 225 230 235 240  
 Val Pro Val Arg Phe Thr Ser Trp Met Gly Gly Asp Arg Asp Gly Asn  
 245 250 255  
 30 Pro Asn Val Thr Ala Asp Ile Thr Arg His Val Leu Leu Leu Ser Arg  
 260 265 270  
 Trp Lys Ala Thr Asp Leu Phe Leu Lys Asp Ile Gln Val Leu Val Ser  
 275 280 285  
 35 Glu Leu Ser Met Val Glu Ala Thr Pro Glu Leu Leu Ala Leu Val Gly  
 290 295 300  
 Glu Glu Gly Ala Ala Glu Pro Tyr Arg Tyr Leu Met Lys Asn Leu Arg  
 305 310 315 320  
 Ser Arg Leu Met Ala Thr Gln Ala Trp Leu Glu Ala Arg Leu Lys Gly  
 325 330 335  
 40 Glu Glu Leu Pro Lys Pro Glu Gly Leu Leu Thr Gln Asn Glu Glu Leu  
 340 345 350  
 Trp Glu Pro Leu Tyr Ala Cys Tyr Gln Ser Leu Gln Ala Cys Gly Met  
 355 360 365  
 45 Gly Ile Ile Ala Asn Gly Asp Leu Leu Asp Thr Leu Arg Arg Val Lys  
 370 375 380  
 Cys Phe Gly Val Pro Leu Val Arg Ile Asp Ile Arg Gln Glu Ser Thr  
 385 390 395 400  
 50 Arg His Thr Glu Ala Leu Gly Glu Leu Thr Arg Tyr Leu Gly Ile Gly  
 405 410 415  
 Asp Tyr Glu Ser Trp Ser Glu Ala Asp Lys Gln Ala Phe Leu Ile Arg

				420					425				430
	Glu	Leu	Asn	Ser	Lys	Arg	Pro	Leu	Leu	Pro	Arg	Asn	Trp
			435					440				445	
5	Ala	Glu	Thr	Arg	Glu	Val	Leu	Asp	Thr	Cys	Gln	Val	Ile
		450					455				460		
	Pro	Gln	Gly	Ser	Ile	Ala	Ala	Tyr	Val	Ile	Ser	Met	Ala
	465				470				475				480
10	Ser	Asp	Val	Leu	Ala	Val	His	Leu	Leu	Leu	Lys	Glu	Ala
			485					490				495	
	Phe	Ala	Met	Pro	Val	Ala	Pro	Leu	Phe	Glu	Thr	Leu	Asp
			500					505				510	
15	Asn	Ala	Asn	Asp	Val	Met	Thr	Gln	Leu	Leu	Asn	Ile	Asp
		515						520				525	
	Gly	Leu	Ile	Gln	Gly	Lys	Gln	Met	Val	Met	Ile	Gly	Tyr
	530						535				540		
20	Ala	Lys	Asp	Ala	Gly	Val	Met	Ala	Ala	Ser	Trp	Ala	Gln
	545					550				555			560
	Gln	Asp	Ala	Leu	Ile	Lys	Thr	Cys	Glu	Lys	Ala	Gly	Ile
			565					570				575	
25	Leu	Phe	His	Gly	Arg	Gly	Gly	Ser	Ile	Gly	Arg	Gly	Gly
			580					585				590	
	His	Ala	Ala	Leu	Leu	Ser	Gln	Pro	Pro	Gly	Ser	Leu	Lys
		595					600					605	
	Arg	Val	Thr	Glu	Gln	Gly	Glu	Met	Ile	Arg	Phe	Lys	Tyr
	610					615					620		
30	Glu	Ile	Thr	Val	Ser	Ser	Leu	Ser	Leu	Tyr	Thr	Gly	Ala
	625				630					635			640
	Ala	Asn	Leu	Leu	Pro	Pro	Glu	Pro	Lys	Glu	Ser	Trp	Arg
			645					650				655	
35	Met	Asp	Glu	Leu	Ser	Val	Ile	Ser	Cys	Asp	Val	Tyr	Arg
		660						665				670	
	Arg	Glu	Asn	Lys	Asp	Phe	Val	Pro	Tyr	Phe	Arg	Ser	Ala
		675				680					685		
40	Gln	Glu	Leu	Gly	Lys	Leu	Pro	Leu	Gly	Ser	Arg	Pro	Ala
	690					695					700		
	Pro	Thr	Gly	Gly	Val	Glu	Ser	Leu	Arg	Ala	Ile	Pro	Trp
	705				710				715				720
45	Trp	Thr	Gln	Asn	Arg	Leu	Met	Leu	Pro	Ala	Trp	Leu	Gly
			725					730				735	
	Ala	Leu	Gln	Lys	Val	Val	Glu	Asp	Gly	Lys	Gln	Ser	Glu
			740					745				750	
	Met	Cys	Arg	Asp	Trp	Pro	Phe	Phe	Ser	Thr	Arg	Leu	Gly
		755				760					765		
50	Met	Val	Phe	Ala	Lys	Ala	Asp	Leu	Trp	Leu	Ala	Glu	Tyr
		770				775					780		

Arg Leu Val Asp Lys Ala Leu Trp Pro Leu Gly Lys Glu Leu Arg Asn  
 785 790 795 800  
 Leu Gln Glu Glu Asp Ile Lys Val Val Leu Ala Ile Ala Asn Asp Ser  
 5 805 810 815  
 His Leu Met Ala Asp Leu Pro Trp Ile Ala Glu Ser Ile Gln Leu Arg  
 820 825 830  
 Asn Ile Tyr Thr Asp Pro Leu Asn Val Leu Gln Ala Glu Leu Leu His  
 10 835 840 845  
 Arg Ser Arg Gln Ala Glu Lys Glu Gly Gln Glu Pro Asp Pro Arg Val  
 850 855 860  
 Glu Gln Ala Leu Met Val Thr Ile Ala Gly Ile Ala Ala Gly Met Arg  
 15 865 870 875 880  
 Asn Thr Gly

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TOGGAAGTA GCACTGTCA CTT

23

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACGGAATTCA ATCTACGGC C

21

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1643  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: genomic DNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: *Corynebacterium glutamicum*

(C) STRAIN: ATCC13869

## (ix) FEATURE:

(A) NAME/KEY: mat peptide

(B) LOCATION: 217..1482

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

10	TCGGGAAGTA GCACCTGTCA CTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC	60
	TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAAOCCTGT	120
	GCAGAAAGAA AACACTOCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG	180
	GTAAGTGTCA GCACGTAGAT CGAAAGGTGC ACAAAG GTG GCC CTG GTC GTA CAG	234
	Met Ala Leu Val Val Gln	
15	1 5	
	AAA TAT GGC GGT TOC TOG CTT GAG AGT GOG GAA CGC ATT AGA AAC GTC	282
	Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala Glu Arg Ile Arg Asn Val	
	10 15 20	
20	GCT GAA CGG ATC GTT GCC ACC AAG AAG GCT GGA AAT GAT GTC GTG GTT	330
	Ala Glu Arg Ile Val Ala Thr Lys Lys Ala Gly Asn Asp Val Val Val	
	25 30 35	
	GTC TGC TOC GCA ATG GGA GAC ACC ACG GAT GAA CTT CTA GAA CTT GCA	378
	Val Cys Ser Ala Met Gly Asp Thr Thr Asp Glu Leu Leu Glu Ala	
25	40 45 50	
	GOG GCA GTG AAT CCC GTT CCG CCA GCT OGT GAA ATG GAT ATG CTC CTG	426
	Ala Ala Val Asn Pro Val Pro Pro Ala Arg Glu Met Asp Met Leu Leu	
	55 60 65 70	
30	ACT GCT GGT GAG CGT ATT TCT AAC GCT CTC GTC GCC ATG GCT ATT GAG	474
	Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu Val Ala Met Ala Ile Glu	
	75 80 85	
	TOC CTT GGC GCA GAA GCT CAA TCT TTC ACT GGC TCT CAG GCT GGT GTG	522
	Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr Gly Ser Gln Ala Gly Val	
35	90 95 100	
	CTC ACC ACC GAG CGC CAC GGA AAC GCA CGC ATT GTT GAC GTC ACA CCG	570
	Leu Thr Thr Glu Arg His Gly Asn Ala Arg Ile Val Asp Val Thr Pro	
	105 110 115	
40	GGT CGT GTG CGT GAA GCA CTC GAT GAG GGC AAG ATC TGC ATT GTT GCT	618
	Gly Arg Val Arg Glu Ala Leu Asp Glu Gly Lys Ile Cys Ile Val Ala	
	120 125 130	
	GGT TTT CAG GGT GTT AAT AAA GAA ACC CGC GAT GTC ACC ACG TTG GGT	666
	Gly Phe Gln Gly Val Asn Lys Glu Thr Arg Asp Val Thr Thr Leu Gly	
45	135 140 145 150	
	CGT GGT GGT TCT GAC ACC ACT GCA GTT GOG TTG GCA GCT GCT TTG AAC	714
	Arg Gly Gly Ser Asp Thr Thr Ala Val Ala Leu Ala Ala Ala Leu Asn	
	155 160 165	

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	GCT	GAT	GTG	TGT	GAG	ATT	TAC	TCG	GAC	GTT	GAC	GGT	GTG	TAT	ACC	GCT	762
	Ala	Asp	Val	Cys	Glu	Ile	Tyr	Ser	Asp	Val	Asp	Gly	Val	Tyr	Thr	Ala	
5				170					175					180			
	GAC	CCG	CGC	ATC	GTT	CCT	AAT	GCA	CAG	AAG	CTG	GAA	AAG	CTC	AGC	TTC	810
	Asp	Pro	Arg	Ile	Val	Pro	Asn	Ala	Gln	Lys	Leu	Glu	Lys	Leu	Ser	Phe	
				185				190						195			
10	GAA	GAA	ATG	CTG	GAA	CTT	GCT	GCT	GTT	GGC	TCC	AAG	ATT	TTG	GTG	CTG	858
	Glu	Glu	Met	Leu	Glu	Leu	Ala	Ala	Val	Gly	Ser	Lys	Ile	Leu	Val	Leu	
				200			205					210					
	CGC	AGT	GTT	GAA	TAC	GCT	CGT	GCA	TTC	AAT	GTG	CCA	CTT	CGC	GTA	CGC	906
	Arg	Ser	Val	Glu	Tyr	Ala	Arg	Ala	Phe	Asn	Val	Pro	Leu	Arg	Val	Arg	
15				215		220					225				230		
	TCG	TCT	TAT	AGT	AAT	GAT	CCC	GGC	ACT	TTG	ATT	GCC	GGC	TCT	ATG	GAG	954
	Ser	Ser	Tyr	Ser	Asn	Asp	Pro	Gly	Thr	Leu	Ile	Ala	Gly	Ser	Met	Glu	
				235						240					245		
20	GAT	ATT	CCT	GTG	GAA	GAA	GCA	GTC	CTT	ACC	GGT	GTC	GCA	ACC	GAC	AAG	1002
	Asp	Ile	Pro	Val	Glu	Glu	Ala	Val	Leu	Thr	Gly	Val	Ala	Thr	Asp	Lys	
				250				255						260			
	TCC	GAA	GCC	AAA	GTA	ACC	GTT	CTG	GGT	ATT	TCC	GAT	AAG	CCA	GCC	GAG	1050
	Ser	Glu	Ala	Lys	Val	Thr	Val	Leu	Gly	Ile	Ser	Asp	Lys	Pro	Gly	Glu	
				265			270							275			
25	GCT	GCC	AAG	GTT	TTC	CGT	GCG	TTG	GCT	GAT	GCA	GAA	ATC	AAC	ATT	GAC	1098
	Ala	Ala	Lys	Val	Phe	Arg	Ala	Leu	Ala	Asp	Ala	Glu	Ile	Asn	Ile	Asp	
				280			285					290					
30	ATG	GTT	CTG	CAG	AAC	GTC	TCC	TCT	GTG	GAA	GAC	GGC	ACC	ACC	GAC	ATC	1146
	Met	Val	Leu	Gln	Asn	Val	Ser	Ser	Val	Glu	Asp	Gly	Thr	Thr	Asp	Ile	
				295		300					305				310		
	ACG	TTC	ACC	TGC	CCT	CGC	GCT	GAC	GGA	CGC	CGT	GCG	ATG	GAG	ATC	TTG	1194
	Thr	Phe	Thr	Cys	Pro	Arg	Ala	Asp	Gly	Arg	Arg	Ala	Met	Glu	Ile	Leu	
				315						320				325			
35	AAG	AAG	CTT	CAG	GTT	CAG	GGC	AAC	TGG	ACC	AAT	GTG	CTT	TAC	GAC	GAC	1242
	Lys	Lys	Leu	Gln	Val	Gln	Gly	Asn	Trp	Thr	Asn	Val	Leu	Tyr	Asp	Asp	
				330				335						340			
40	CAG	GTC	GGC	AAA	GTC	TCC	CTC	GTG	GGT	GCT	GGC	ATG	AAG	TCT	CAC	CCA	1290
	Gln	Val	Gly	Lys	Val	Ser	Leu	Val	Gly	Ala	Gly	Met	Lys	Ser	His	Pro	
				345			350						355				
	GGT	GTT	ACC	GCA	GAG	TTC	ATG	GAA	GCT	CTG	CGC	GAT	GTC	AAC	GTG	AAC	1338
	Gly	Val	Thr	Ala	Glu	Phe	Met	Glu	Ala	Leu	Arg	Asp	Val	Asn	Val	Asn	
				360			365					370					
45	ATC	GAA	TTG	ATT	TCC	ACC	TCT	GAG	ATC	CGC	ATT	TCC	GTG	CTG	ATC	CGT	1386
	Ile	Glu	Leu	Ile	Ser	Thr	Ser	Glu	Ile	Arg	Ile	Ser	Val	Leu	Ile	Arg	
				375		380					385				390		
	GAA	GAT	GAT	CTG	GAT	GCT	GCT	GCA	CGT	GCA	TTG	CAT	GAG	CAG	TTC	CAG	1434
50	Glu	Asp	Asp	Leu	Asp	Ala	Ala	Ala	Arg	Ala	Leu	His	Glu	Gln	Phe	Gln	
				395						400					405		

CTG GGC GGC GAA GAC GAA GCC GTC GTT TAT GCA GGC ACC GGA CGC TAA 1482  
 Leu Gly Gly Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg  
                   410                  415                  420  
 5 AGTTTAAAG GAGTAGTTT ACAATGACCA CCATCGCAGT TGTGTTGCA ACCGGCCAGG 1542  
 TOGGCCAGGT TATGCGCACC CTTTGGGAAG AGCGCAATTT OCCAGCTGAC ACTGTTGTT 1602  
 TCTTTCCTTC CCGCGTTCC GCAGGCGTA AGATTGAATT C 1643

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 421 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ala Leu Val Val Gln Lys Tyr Gly Gly Ser Ser Leu Glu Ser Ala  
   1                  5                  10                  15  
 20 Glu Arg Ile Arg Asn Val Ala Glu Arg Ile Val Ala Thr Lys Lys Ala  
                   20                  25                  30  
 Gly Asn Asp Val Val Val Val Cys Ser Ala Met Gly Asp Thr Thr Asp  
                   35                  40                  45  
 25 Glu Leu Leu Glu Leu Ala Ala Val Asn Pro Val Pro Pro Ala Arg  
                   50                  55                  60  
 Glu Met Asp Met Leu Leu Thr Ala Gly Glu Arg Ile Ser Asn Ala Leu  
   65                  70                  75                  80  
 Val Ala Met Ala Ile Glu Ser Leu Gly Ala Glu Ala Gln Ser Phe Thr  
                   85                  90                  95  
 30 Gly Ser Gln Ala Gly Val Leu Thr Thr Glu Arg His Gly Asn Ala Arg  
                   100                  105                  110  
 Ile Val Asp Val Thr Pro Gly Arg Val Arg Glu Ala Leu Asp Glu Gly  
                   115                  120                  125  
 35 Lys Ile Cys Ile Val Ala Gly Phe Gln Gly Val Asn Lys Glu Thr Arg  
                   130                  135                  140  
 Asp Val Thr Thr Leu Gly Arg Gly Gly Ser Asp Thr Thr Ala Val Ala  
   145                  150                  155                  160  
 40 Leu Ala Ala Ala Leu Asn Ala Asp Val Cys Glu Ile Tyr Ser Asp Val  
                   165                  170                  175  
 Asp Gly Val Tyr Thr Ala Asp Pro Arg Ile Val Pro Asn Ala Gln Lys  
                   180                  185                  190  
 45 Leu Glu Lys Leu Ser Phe Glu Glu Met Leu Glu Leu Ala Ala Val Gly  
                   195                  200                  205  
 Ser Lys Ile Leu Val Leu Arg Ser Val Glu Tyr Ala Arg Ala Phe Asn  
                   210                  215                  220  
 50 Val Pro Leu Arg Val Arg Ser Ser Tyr Ser Asn Asp Pro Gly Thr Leu  
                   225                  230                  235                  240

Ile Ala Gly Ser Met Glu Asp Ile Pro Val Glu Glu Ala Val Leu Thr  
 245 250 255  
 Gly Val Ala Thr Asp Lys Ser Glu Ala Lys Val Thr Val Leu Gly Ile  
 260 265 270  
 Ser Asp Lys Pro Gly Glu Ala Ala Lys Val Phe Arg Ala Leu Ala Asp  
 275 280 285  
 Ala Glu Ile Asn Ile Asp Met Val Leu Gln Asn Val Ser Ser Val Glu  
 290 295 300  
 Asp Gly Thr Thr Asp Ile Thr Phe Thr Cys Pro Arg Ala Asp Gly Arg  
 305 310 315 320  
 Arg Ala Met Glu Ile Leu Lys Lys Leu Gln Val Gln Gly Asn Trp Thr  
 325 330 335  
 Asn Val Leu Tyr Asp Asp Gln Val Gly Lys Val Ser Leu Val Gly Ala  
 340 345 350  
 Gly Met Lys Ser His Pro Gly Val Thr Ala Glu Phe Met Glu Ala Leu  
 355 360 365  
 Arg Asp Val Asn Val Asn Ile Glu Leu Ile Ser Thr Ser Glu Ile Arg  
 370 375 380  
 Ile Ser Val Leu Ile Arg Glu Asp Asp Leu Asp Ala Ala Ala Arg Ala  
 385 390 395 400  
 Leu His Glu Gln Phe Gln Leu Gly Gly Glu Asp Glu Ala Val Val Tyr  
 405 410 415  
 Ala Gly Thr Gly Arg  
 420

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1643
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## (ii) MOLECULAR TYPE: genomic DNA

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Corynebacterium glutamicum*
- (C) STRAIN: ATCC13869

## (ix) FEATURE:

- (A) NAME/KEY: mat peptide
- (B) LOCATION: 964..1482

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCGCGAAGTA	GCACTGTCA	CTTTGTCTC	AAATATTAA	TCGAATATCA	ATATACGGTC	60
TGTTTATTGG	AACGCATCC	AGTGGCTGAG	ACGCATCCG	TAAAGCCCCA	GGAAACCTGT	120
GCAGAAAGAA	AACACTCTC	TGGCTAGGTA	GACACAGTTT	ATAAAGGTAG	AGTTGAGCGG	180
GTAAGTGTCA	GCACTAGAT	CGAAAGGTGC	ACAAAGGTGG	CCCTGGTCTG	ACAGAAATAT	240



	GGCGGTTCCT	CGCTTGAGAG	TGCGGAAOCG	ATTAGAAACG	TOGCTGAACG	GATCGTTGCC	300										
	ACCAAGAAGG	CTGGAAATGA	TGTOGTGGTT	GTCTGCTCOG	CAATGGGAGA	CACCAOGGAT	360										
5	GAACCTTCTAG	AACTTGCAGC	GGCAGTGAAT	CCCGTTCCGC	CAGCTCGTGA	AATGGATATG	420										
	CTOCTGACTG	CTGGTGAGCG	TATTTCTAAC	GCTCTCGTOG	CCATGGCTAT	TGAGTCCCTT	480										
	GGCGCAGAAG	CTCAATCTTT	CACTGGCTCT	CAGGCTGGTG	TGCTCAOCCAC	CGAGCGOCAC	540										
	GGAAACGCAC	GCAATTGTTGA	CGTCACACCG	GGTCGTGTGC	GTGAAGCACT	CGATGAGGGC	600										
	AAGATCTGCA	TTGTTGCTGG	TTTTTCAGGGT	GTTAATAAAG	AAACCCGOGA	TGTCAOCCAG	660										
10	TTGGGTGCTG	GTGGTTCTGA	CACCACTGCA	GTTCGGTTGG	CAGCTGCTTT	GAACGCTGAT	720										
	GTGTGTGAGA	TTTACTOGGA	CGTTGACGGT	GTGTATAOCC	CTGAOCCGCG	CATOGTTCT	780										
	AATGCACAGA	AGCTGGAAAA	GCTCAGCTTC	GAAGAAATGC	TGGAACCTGC	TGCTGTTGGC	840										
	TOCAAGATTT	TGGTGCTGCG	CAGTGTGAA	TACGCTCGTG	CATTCAATGT	GCCACTTCGC	900										
15	GTACGCTCGT	CTTATAGTAA	TGATCCCGGC	ACTTTGATTG	CCGGCTCTAT	GGAGGATATT	960										
	CCT	GTG	GAA	GAA	GCA	GTC	CTT	ACC	GGT	GTC	GCA	ACC	GAC	AAG	TOC	GAA	1008
		Met	Glu	Glu	Ala	Val	Leu	Thr	Gly	Val	Ala	Thr	Asp	Lys	Ser	Glu	
		1				5					10				15		
	GCC	AAA	GTA	ACC	GTT	CTG	GGT	ATT	TOC	GAT	AAG	CCA	GGC	GAG	GCT	GOC	1056
20	Ala	Lys	Val	Thr	Val	Leu	Gly	Ile	Ser	Asp	Lys	Pro	Gly	Glu	Ala	Ala	
					20					25					30		
	AAG	GTT	TTC	CGT	GCG	TTG	GCT	GAT	GCA	GAA	ATC	AAC	ATT	GAC	ATG	GTT	1104
	Lys	Val	Phe	Arg	Ala	Leu	Ala	Asp	Ala	Glu	Ile	Asn	Ile	Asp	Met	Val	
				35					40					45			
25	CTG	CAG	AAC	GTC	TOC	TCT	GTG	GAA	GAC	GGC	ACC	ACC	GAC	ATC	ACG	TTC	1152
	Leu	Gln	Asn	Val	Ser	Ser	Val	Glu	Asp	Gly	Thr	Thr	Asp	Ile	Thr	Phe	
				50				55					60				
	ACC	TGC	OCT	CGC	GCT	GAC	GGA	CGC	CGT	GCG	ATG	GAG	ATC	TTG	AAG	AAG	1200
30	Thr	Cys	Pro	Arg	Ala	Asp	Gly	Arg	Arg	Ala	Met	Glu	Ile	Leu	Lys	Lys	
		65					70					75					
	CTT	CAG	GTT	CAG	GGC	AAC	TGG	ACC	AAT	GTG	CTT	TAC	GAC	GAC	CAG	GTC	1248
	Leu	Gln	Val	Gln	Gly	Asn	Trp	Thr	Asn	Val	Leu	Tyr	Asp	Asp	Gln	Val	
	80					85					90				95		
35	GGC	AAA	GTC	TOC	CTC	GTG	GGT	GCT	GGC	ATG	AAG	TCT	CAC	OCA	GGT	GTT	1296
	Gly	Lys	Val	Ser	Leu	Val	Gly	Ala	Gly	Met	Lys	Ser	His	Pro	Gly	Val	
				100					105					110			
	ACC	GCA	GAG	TTC	ATG	GAA	GCT	CTG	CGC	GAT	GTC	AAC	GTG	AAC	ATC	GAA	1344
40	Thr	Ala	Glu	Phe	Met	Glu	Ala	Leu	Arg	Asp	Val	Asn	Val	Asn	Ile	Glu	
				115					120					125			
	TTG	ATT	TOC	ACC	TCT	GAG	ATC	CGC	ATT	TOC	GTG	CTG	ATC	CGT	GAA	GAT	1392
	Leu	Ile	Ser	Thr	Ser	Glu	Ile	Arg	Ile	Ser	Val	Leu	Ile	Arg	Glu	Asp	
				130				135					140				
45	GAT	CTG	GAT	GCT	GCT	GCA	CGT	GCA	TTG	CAT	GAG	CAG	TTC	CAG	CTG	GGC	1440
	Asp	Leu	Asp	Ala	Ala	Ala	Arg	Ala	Leu	His	Glu	Gln	Phe	Gln	Leu	Gly	
		145					150					155					
	GGC	GAA	GAC	GAA	GCC	GTC	GTT	TAT	GCA	GGC	ACC	GGA	CGC	TAAAGTTTTAA			1490
50	Gly	Glu	Asp	Glu	Ala	Val	Val	Tyr	Ala	Gly	Thr	Gly	Arg				
	160					165					170		172				

AGGAGTAGTT TTACAATGAC CAACATCGCA GTTGTTGGTG CAACCGGACA GGTGGGOCAG 1550  
 GPTATGGCA CCTTTTGGGA AGAGCGCAAT TTCCAGCTG ACACTGTTGG TTTCTTTGCT 1610  
 TCCCCGGTT CGCAGGCGG TAAGATTGAA TTC 1643

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 172 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Glu Glu Ala Val Leu Thr Gly Val Ala Thr Asp Lys Ser Glu Ala  
 1 5 10 15  
 Lys Val Thr Val Leu Gly Ile Ser Asp Lys Pro Gly Glu Ala Ala Lys  
 20 25 30  
 Val Phe Arg Ala Leu Ala Asp Ala Glu Ile Asn Ile Asp Met Val Leu  
 35 40 45  
 Gln Asn Val Ser Ser Val Glu Asp Gly Thr Thr Asp Ile Thr Phe Thr  
 50 55 60  
 Cys Pro Arg Ala Asp Gly Arg Arg Ala Met Glu Ile Leu Lys Lys Leu  
 65 70 75 80  
 Gln Val Gln Gly Asn Trp Thr Asn Val Leu Tyr Asp Asp Gln Val Gly  
 85 90 95  
 Lys Val Ser Leu Val Gly Ala Gly Met Lys Ser His Pro Gly Val Thr  
 100 105 110  
 Ala Glu Phe Met Glu Ala Leu Arg Asp Val Asn Val Asn Ile Glu Leu  
 115 120 125  
 Ile Ser Thr Ser Glu Ile Arg Ile Ser Val Leu Ile Arg Glu Asp Asp  
 130 135 140  
 Leu Asp Ala Ala Ala Arg Ala Leu His Glu Gln Phe Gln Leu Gly Gly  
 145 150 155 160  
 Glu Asp Glu Ala Val Val Tyr Ala Gly Thr Gly Arg  
 165 170

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULAR TYPE: other..synthetic DNA

## (iii) HYPOTHETICAL: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AAAAACCTGC GTTCTC

16

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGACTTAAG GTTACAGGCC

20

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ACTGAATTCC AAATGTCCGC

20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULAR TYPE: other..synthetic DNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AAGTGCAGG CCGTTT

16

Claims

1. A mutant phosphoenolpyruvate carboxylase originating from a microorganism belonging to the genus Escherichia, wherein said mutant phosphoenolpyruvate carboxylase has mutation to desensitize feedback inhibition of the phosphoenolpyruvate carboxylase by aspartic acid.
2. A mutant phosphoenolpyruvate carboxylase according to claim 1, which, in the case of being allowed to exist in cells of a microorganism belonging to the genus Escherichia, gives the cells resistance to a compound having the following properties:
  - it exhibits a growth inhibitory action against a microorganism belonging to the genus Escherichia which produces a wild type phosphoenolpyruvate carboxylase;
  - said growth inhibitory action is recovered by existence of L-glutamic acid or L-aspartic acid; and
  - it inhibits wild type phosphoenolpyruvate carboxylase activity.
3. A mutant phosphoenolpyruvate carboxylase according to claim 2, wherein said compound is selected from 3-bromopyruvate, aspartic acid- $\beta$ -hydrazide and DL-threo- $\beta$ -hydroxyaspartic acid.

4. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 625th glutamic acid with lysine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.
- 5 5. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 222th arginine with histidine and 223th glutamic acid with lysine respectively as counted from the N-terminus of the phosphoenolpyruvate carboxylase.
- 10 6. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 288th serine with phenylalanine, 289th glutamic acid with lysine, 551th methionine with isoleucine and 804th glutamic acid with lysine respectively as counted from the N-terminus of the phosphoenolpyruvate carboxylase.
7. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 867th alanine with threonine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.
- 15 8. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 438th arginine with cysteine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.
9. A mutant phosphoenolpyruvate carboxylase according to claim 1, wherein said mutation is mutation to replace 620th lysine with serine as counted from the N-terminus of the phosphoenolpyruvate carboxylase.
- 20 10. A DNA fragment which codes for the mutant phosphoenolpyruvate carboxylase according to any one of claims 1 to 9.
- 25 11. A microorganism belonging to the genus Escherichia or coryneform bacteria, transformed by allowing the DNA fragment according to claim 10 to be integrated in chromosomal DNA.
12. A recombinant DNA formed by ligating the DNA fragment according to claim 10 with a vector DNA capable of autonomously replication in cells of bacteria belonging to the genus Escherichia or coryneform bacteria.
- 30 13. A microorganism belonging to the genus Escherichia or coryneform bacteria, transformed with the recombinant DNA according to claim 12.
14. A method of producing amino acid, comprising:  
cultivating the microorganism according to claim 11 or 13 in a suitable medium; and  
35 separating, from the medium, an amino acid selected from the group consisting of L-lysine, L-threonine, L-methionine, L-isoleucine, L-glutamic acid, L-arginine and L-proline.

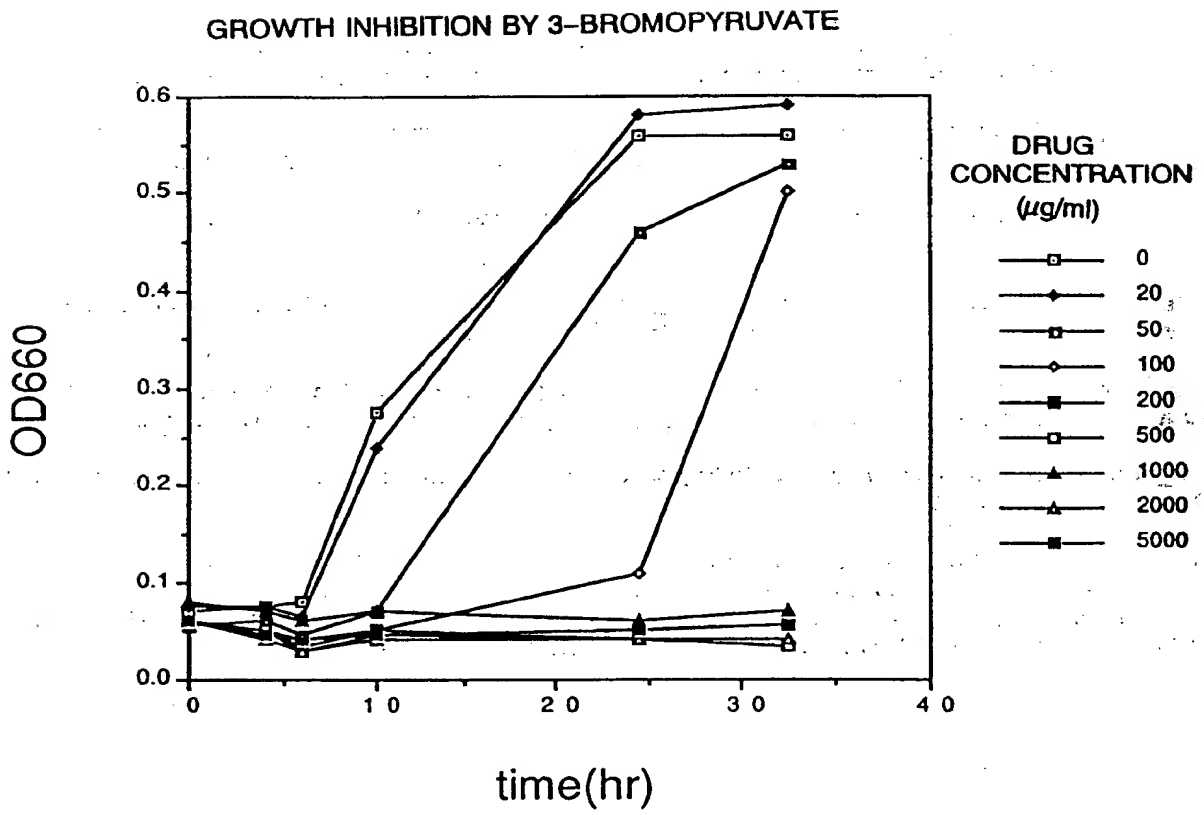


Fig. 1

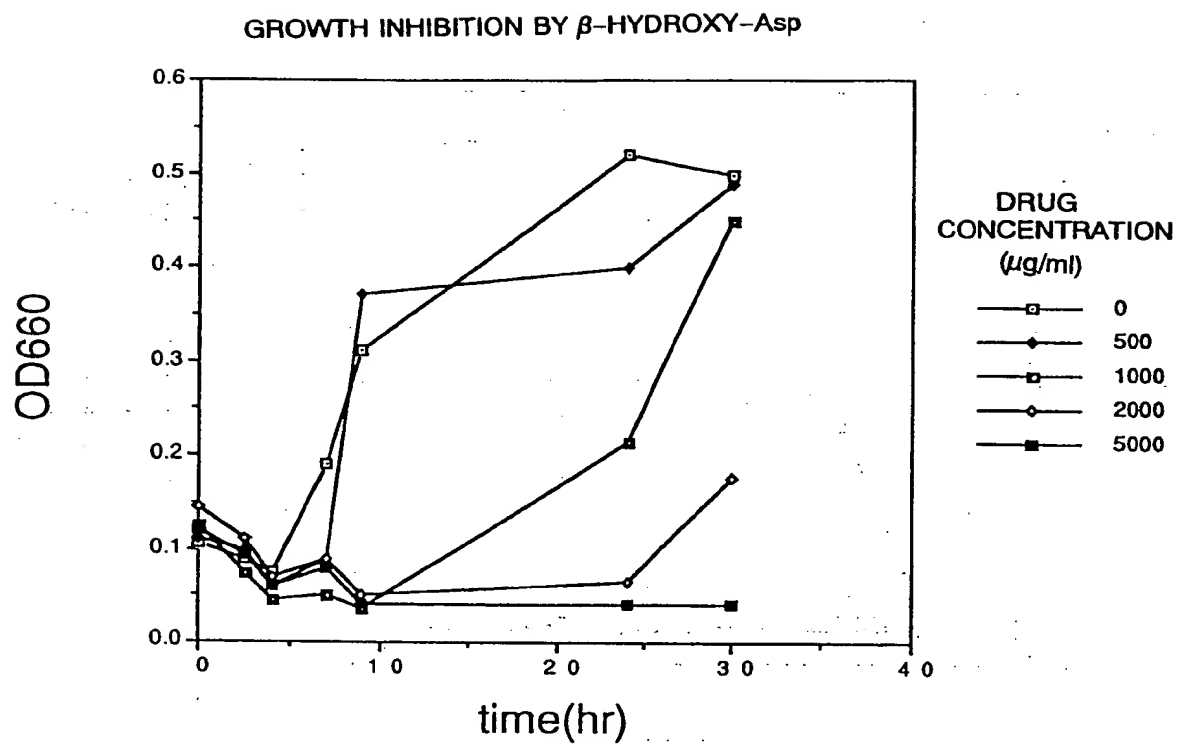


Fig. 2

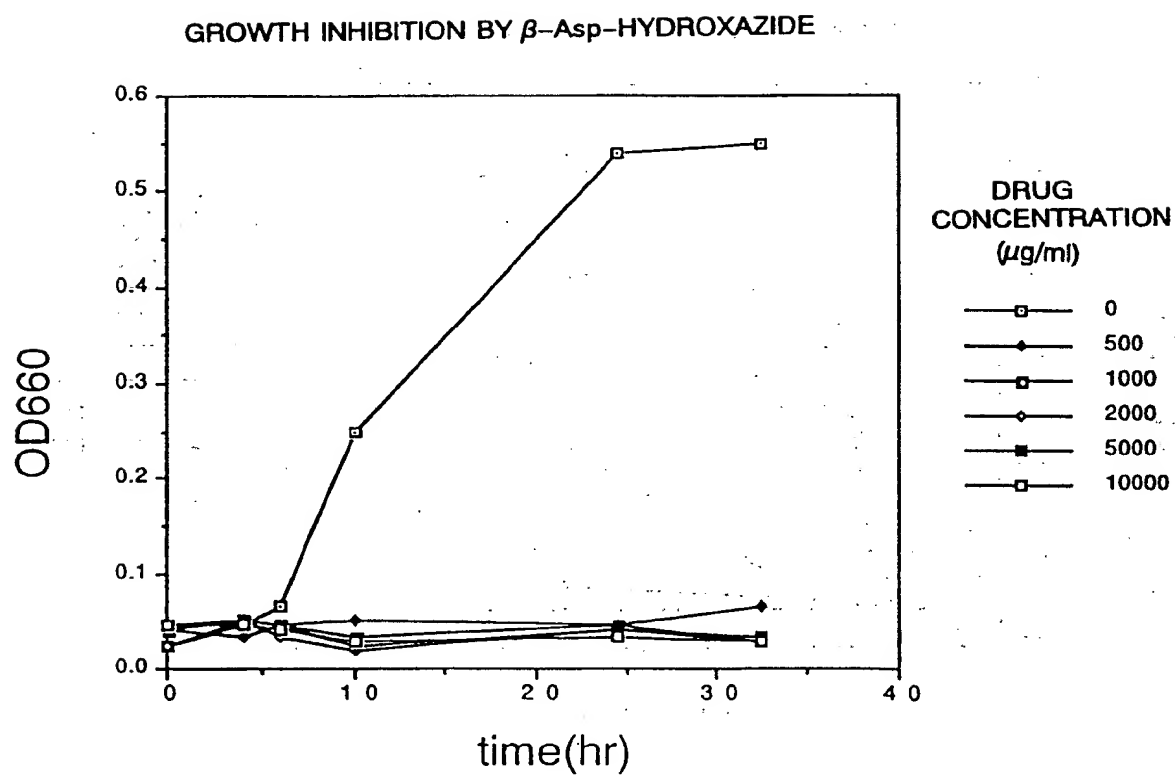


Fig. 3

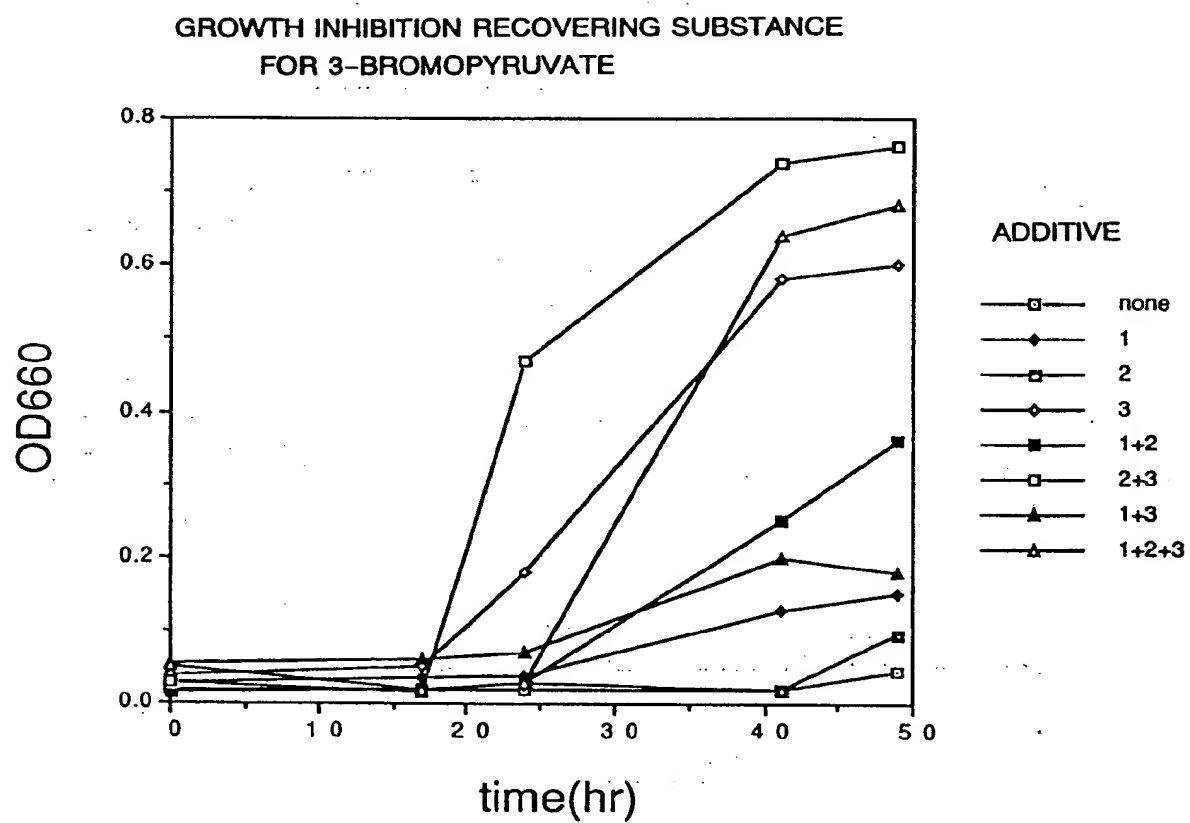


Fig. 4



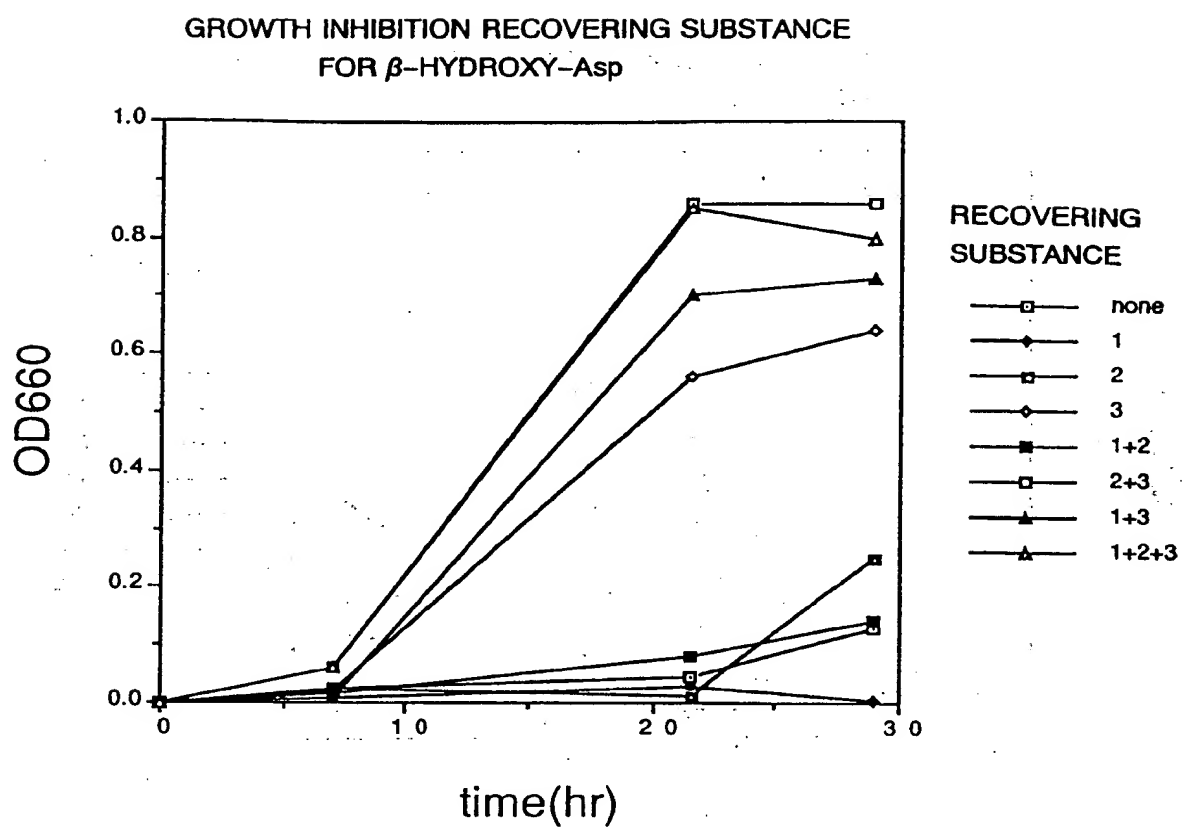


Fig. 5

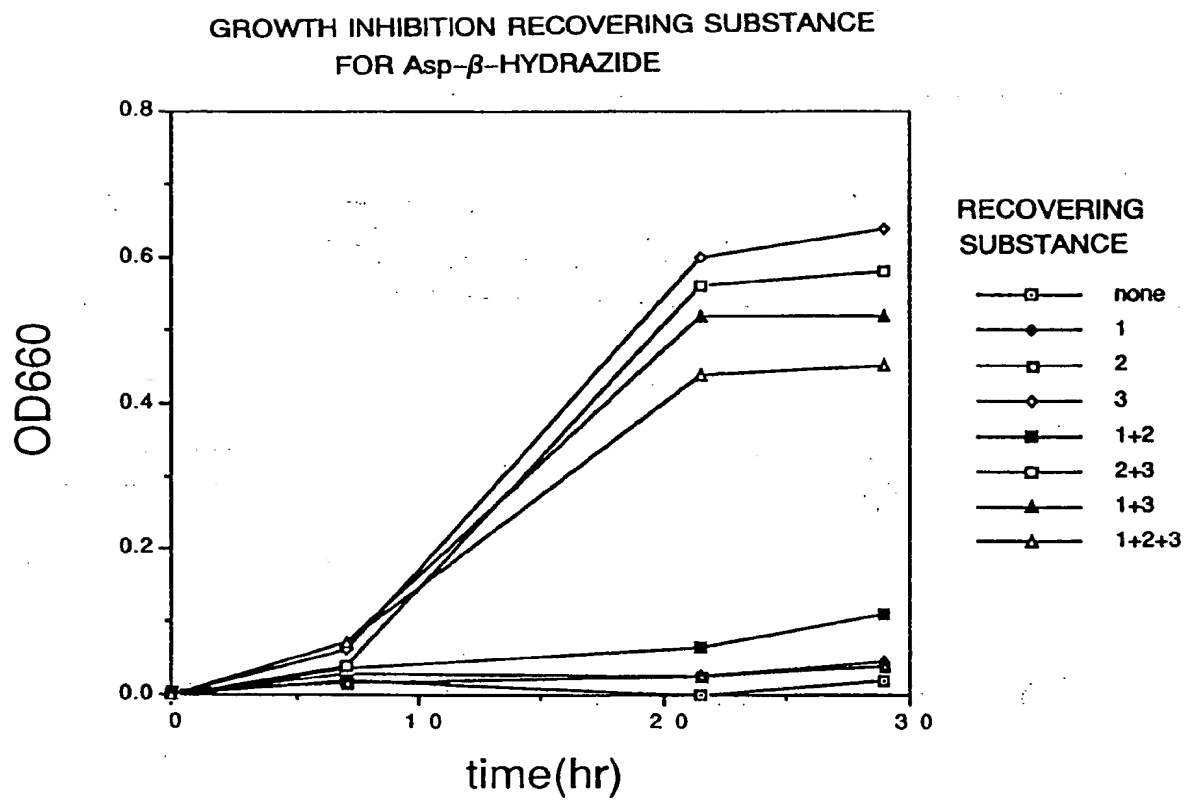


Fig. 6

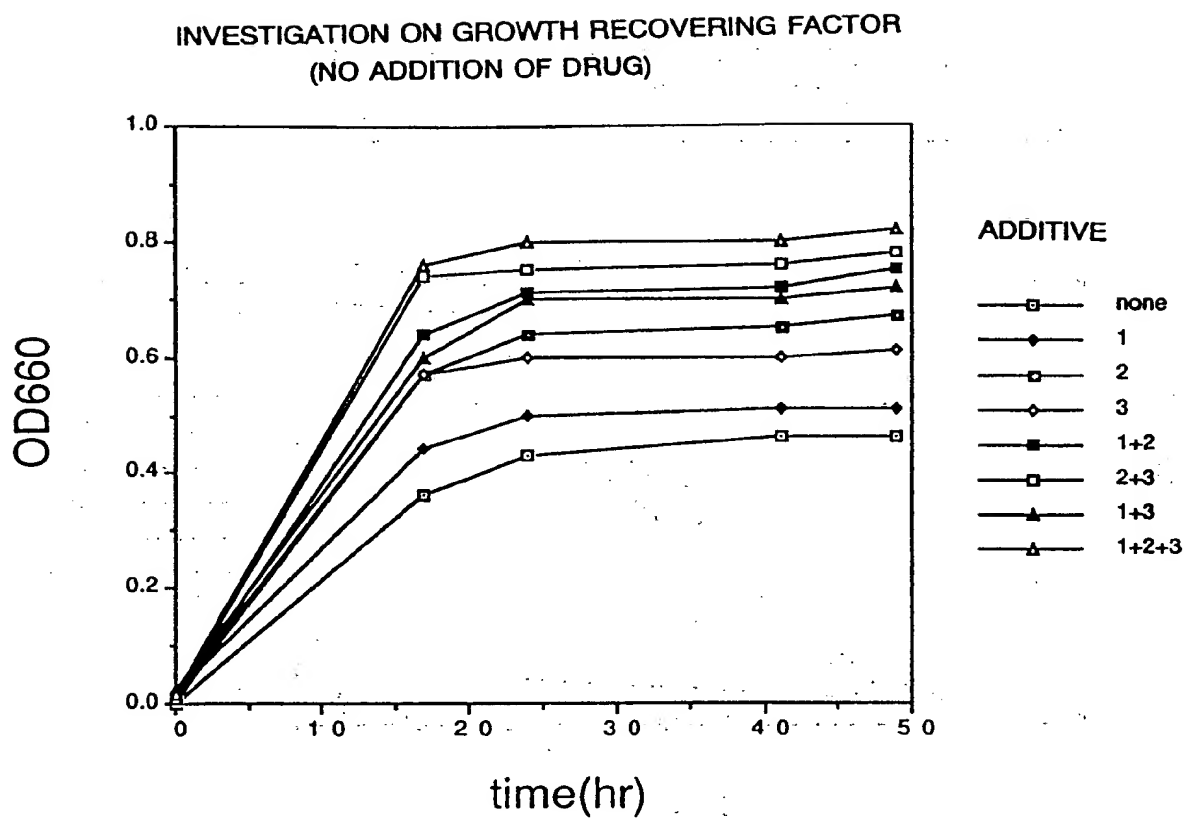


Fig. 7

## INHIBITION OF PEPC ACTIVITY BY SELECTED DRUGS

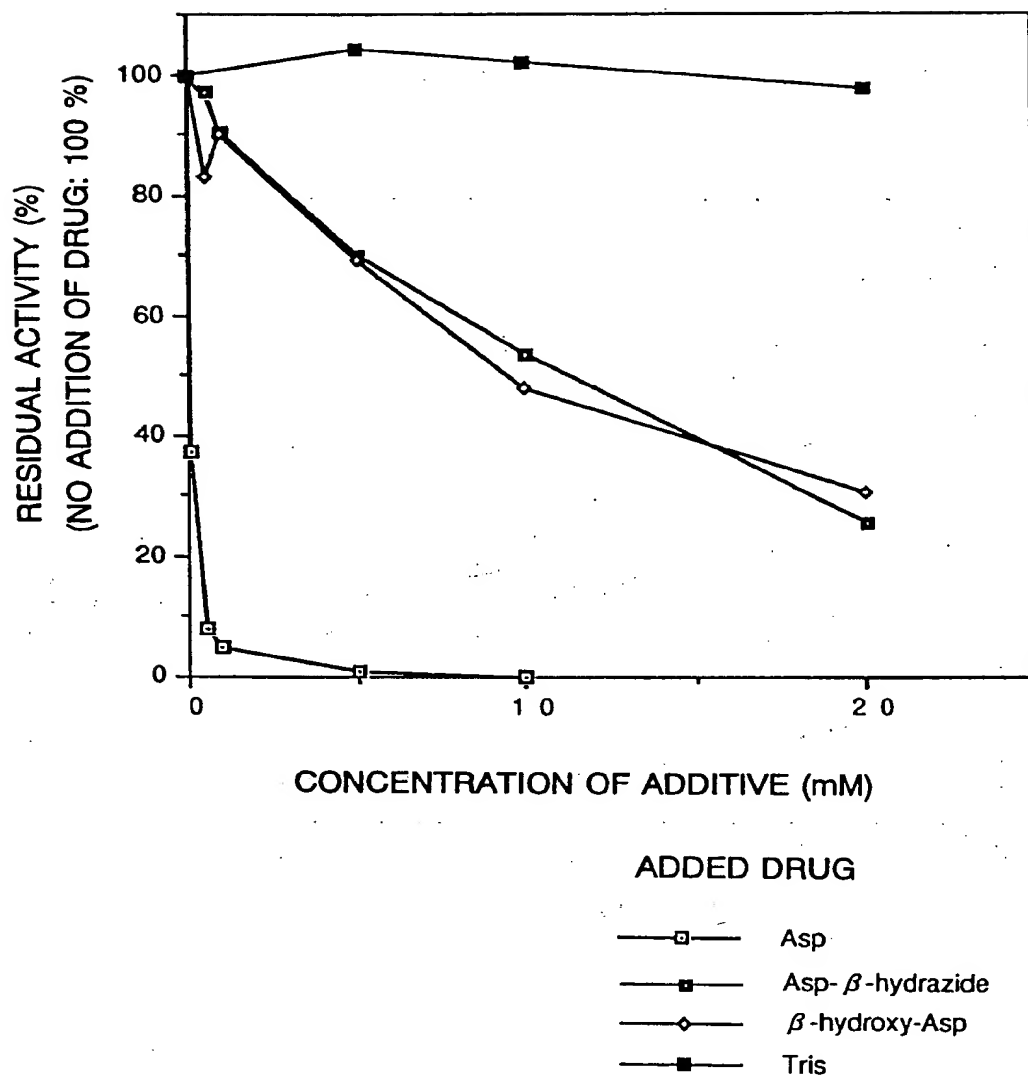


Fig. 8

INHIBITION OF MUTANT TYPE PEPC BY Asp  
(AcCoA: 0.1 mM)

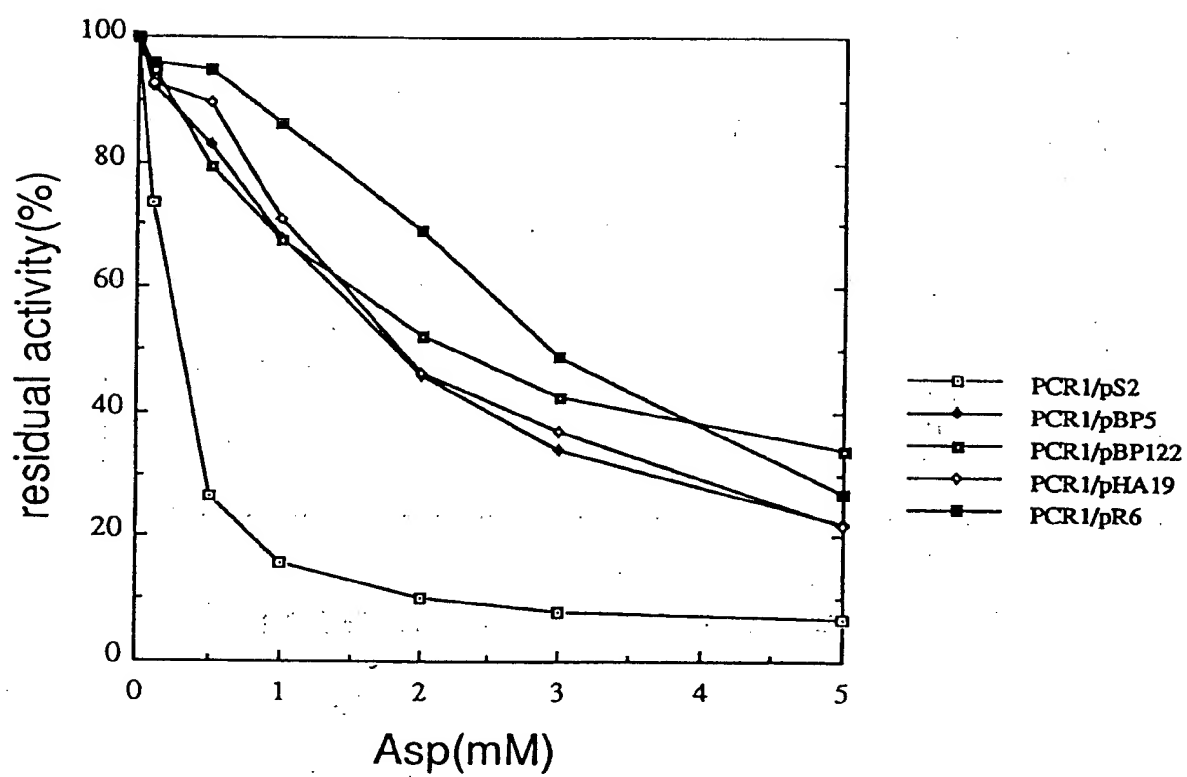


Fig. 9

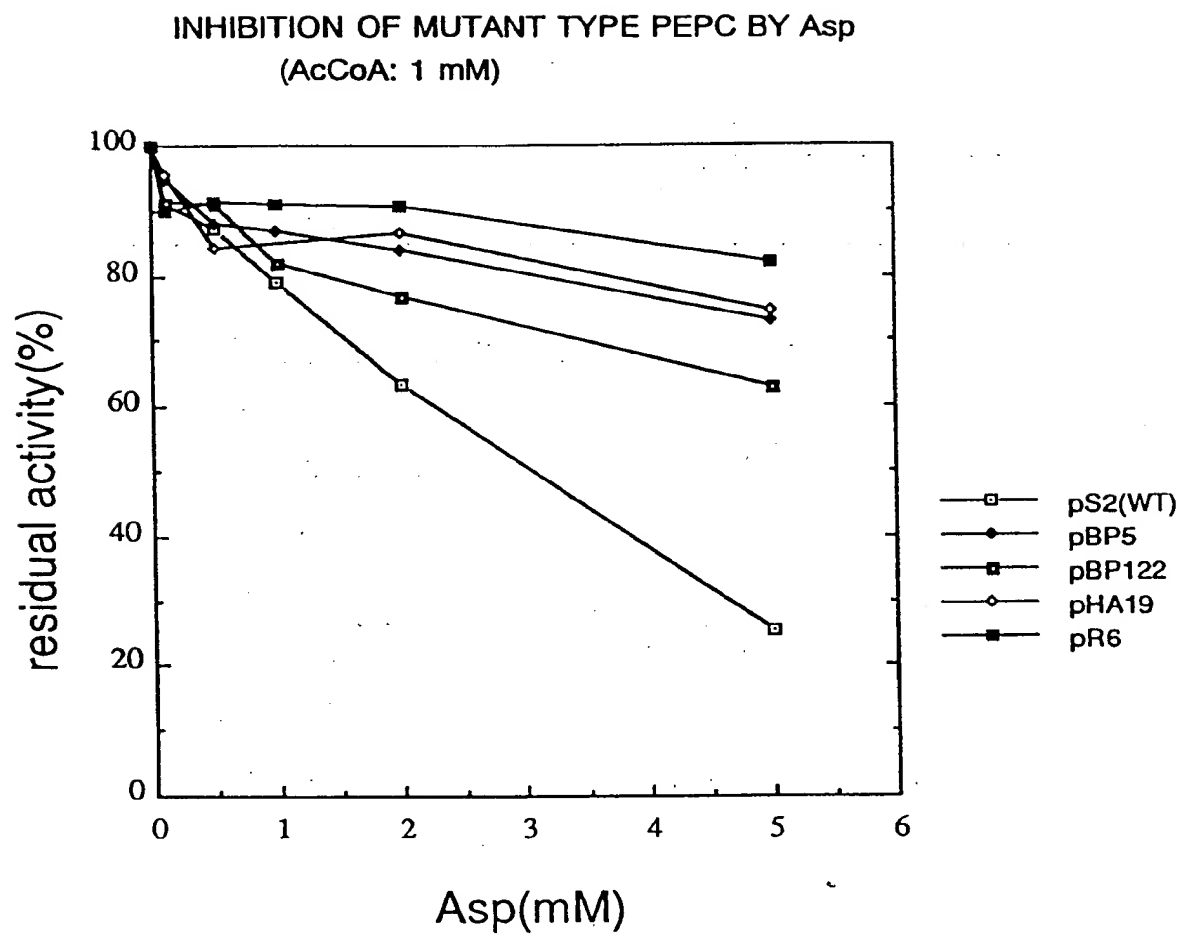


Fig. 10

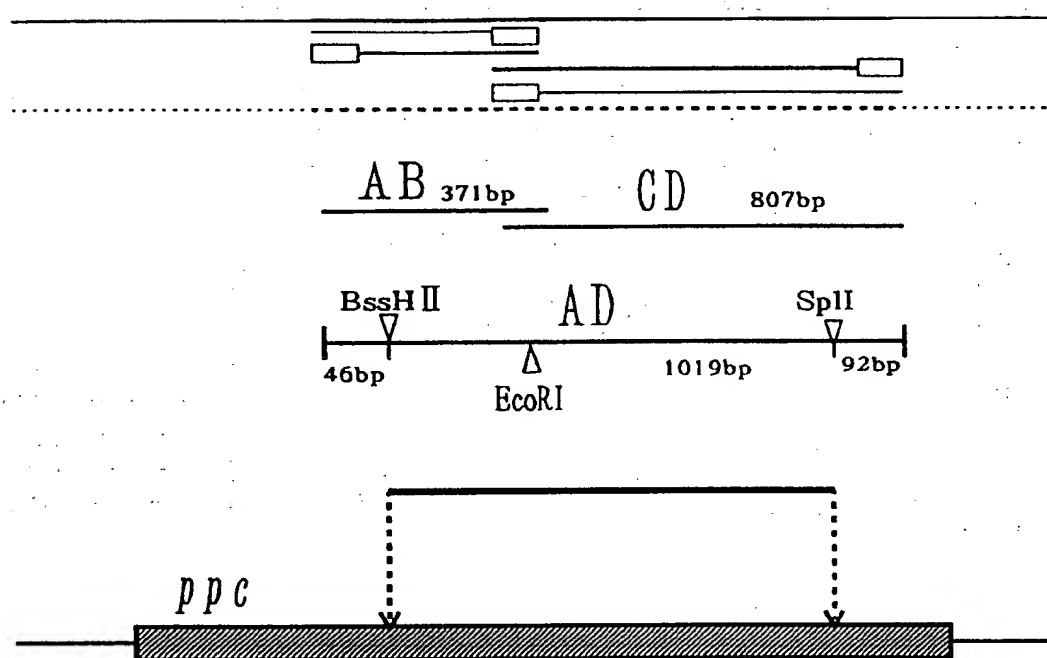


Fig. 11

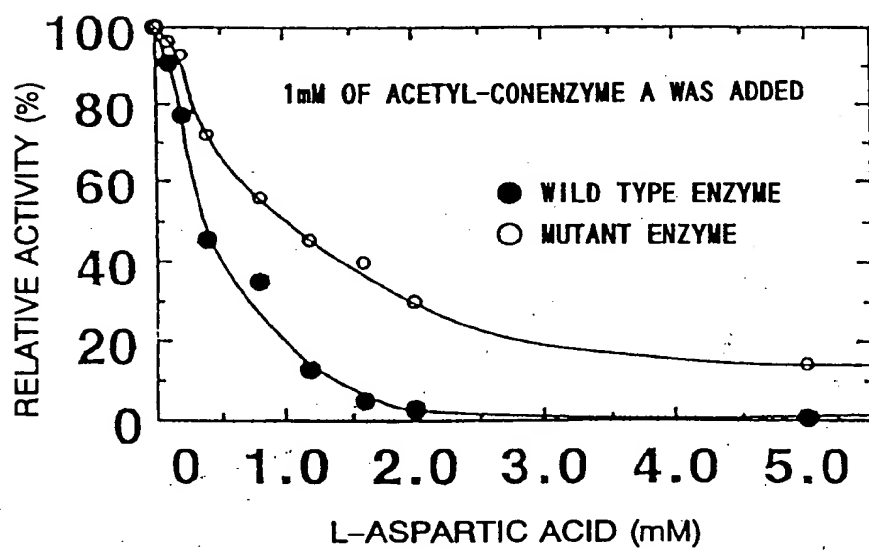
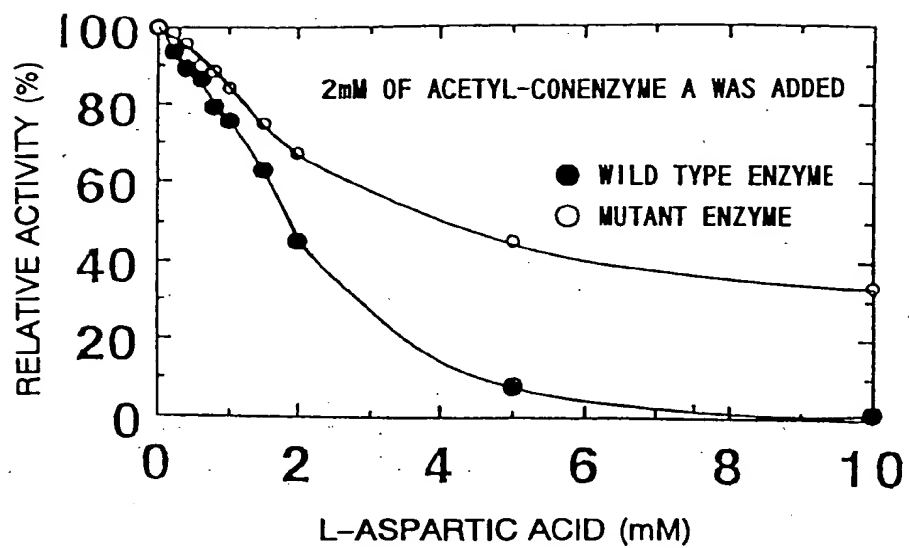


Fig. 12



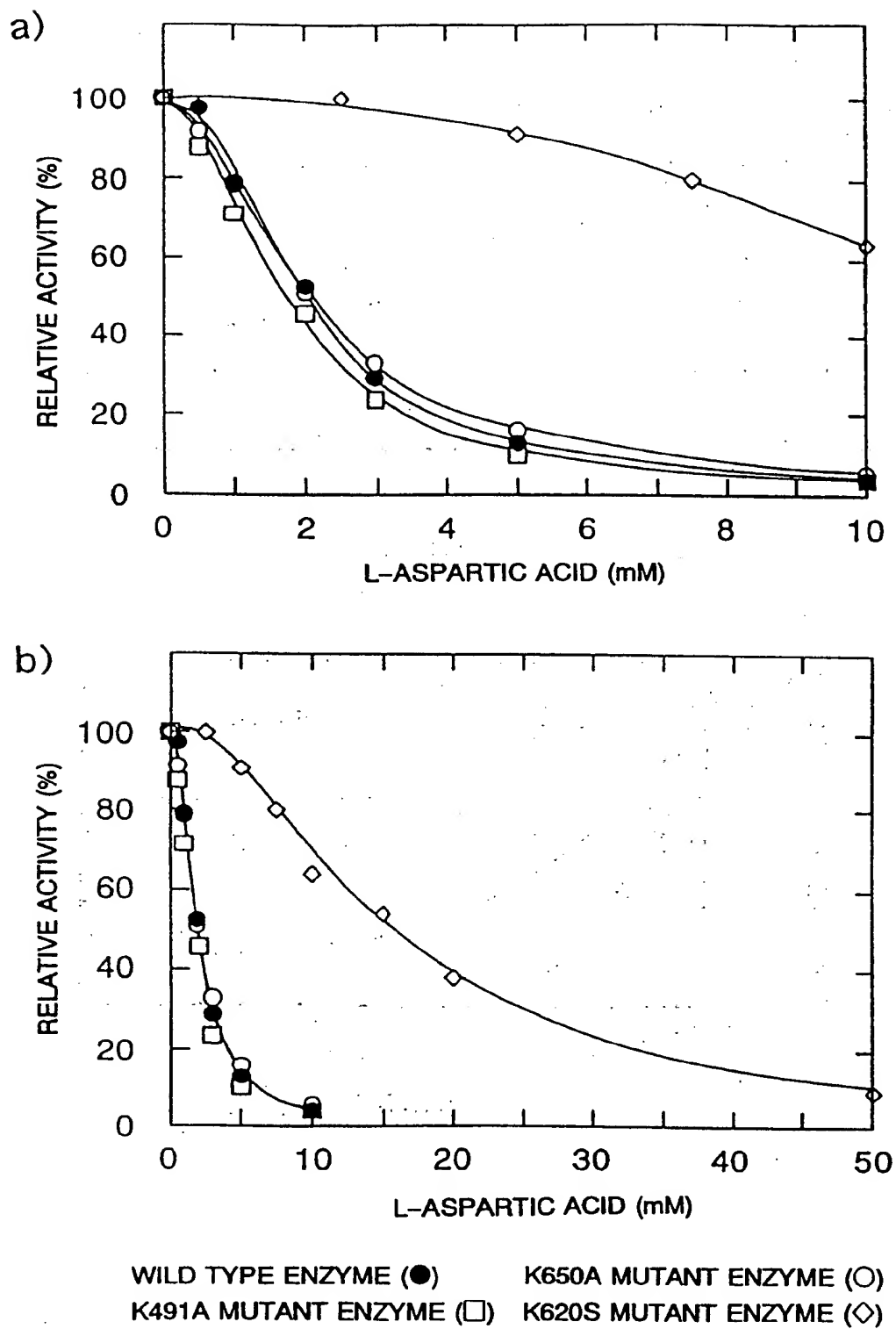


Fig. 13

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP94/01365

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl <sup>6</sup> C12N9/88		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Int. Cl <sup>5</sup> C12N9/88, C12N15/60		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
CAS BIOSIS WPI, WPI/L		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Agric Biol Chem. Vol. 47, No. 7 (1983), Hachiro Ozaki et al. "Production of lysine by pyruvate kinase mutants of Brevibacterium flavum", P. 1569-1576	1, 2, 14
A	J. Biochem. Vol. 95, No. 4 (1984), Fujita Nubuuki et al. "The Primary structure of phosphoenolpyruvate carboxylase of Escherichia coli Nucleotide Sequence of the ppe gene and deduced aminoacid Sequence", P. 909-916	4-13
A	J. Biol Chem. Vol. 265, No. 26 (1990), Sherryl Mowbray et al. "Mutations in the Aspartate Receptor of Escherichia coli Which Affect Aspartate Binding", P. 15638-15643	8
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p>		
Date of the actual completion of the international search		Date of mailing of the international search report
November 1, 1994 (01. 11. 94)		November 22, 1994 (22. 11. 94)
Name and mailing address of the ISA/ Japanese Patent Office		Authorized officer
Facsimile No.		Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)